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Cover Photograph: The Pine Warbler (/Dendroica pinus//)/, a characteristic bird of Alabama pine forests.

Photo courtesy of: Bill Garland, U.S. Fish and Wildlife Service, Biologist, Anniston, Alabama. Photo was taken at Mountain Longleaf National Wildlife Refuge, Alabama.

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CONTENTS

COMPARISON OF METHODS FOR THE EXTRACTION OF GENOMIC DNA FROM AEROBIC DIGEST

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ABSTRACT

Aerobic digest represents a complex microbial community generated by the wastewater treatment process. The development of culture-independent based methods, such as PCR, for detecting microbial pathogens, population monitoring, and tracking of problematic species is dependent upon the isolation of genomic DNA that contains target sequences of sufficient quality and quantity and is free from organic contaminants. Aerobic digest, like soil, contains a concentrated amount of microbial cells in addition to organic pollutants. This study examined several genomic DNA extraction methods to determine which will provide a DNA product free from humic acid contamination and of sufficient quantity to be detected by gel electrophoresis and ethidium bromide staining. Seven genomic DNA extraction methods were tested with four methods (Wizard® Genomic DNA purification, UltraClean™ Microbial DNA Isolation, UltraClean™ Fecal DNA, and UltraClean™ Mega Soil kits) yielding genomic DNA detectable by ethidium bromide gel electrophoresis. 16S rDNA was amplified using the UltraClean™ Fecal DNA samples. The successful PCR reaction used GoTaq® Green polymerase and 45 rounds of amplification. The development of a successful PCR reaction now allows for specific organisms of interest (e.g., pathogens or known sludge bulking species) to be detected early and monitored throughout the aerobic digestion process.

INTRODUCTION

Aerobic digest represents a complex microbial community collected from domestic, industrial and municipal waste streams and generated by the wastewater treatment process itself. A first step to studying this complex community is genomic DNA isolation. However, the majority of microbial species cannot be grown according to current cultivation practices (Kepner, 1994). Once genomic DNA has been isolated, then specific organisms, families, phyla, or domains may be monitored for changes throughout the aerobic digestion process.

Because greater than 90% of bacterial species within a natural sample are noncultival, the only means of studying these organisms and their community interactions is through their genetic or protein material (Atlas and Bartha, 1997). Genetic probes and microarrays

can be developed to determine the presence, absence, or activation of particular genes within a community at either the community level, family level, genus level, or species level (Spano et al., 2005; Siripong et al., 2006). Probes or polymerase chain reaction (PCR) primers can be developed to determine the presence and abundance of species of interest (Dar et al., 2005). However, before the development of probes and tests for the rapid detection of species of concern can be developed, genetic material must be isolated. While there are many protocols that describe DNA isolation from water, soil (Tsai and Olson, 1991), and wastewater (Dar et al., 2005; Spano et al., 2005; Park et al., 2006; Siripong et al., 2006; Lachmayr et al., 2009), most of these protocols fail to isolate a usable DNA product from aerobic digest. During recent years, several groups have investigated the microbial community of activated sludge, an upstream process in wastewater treatment, by using molecular methods such as ribosomal RNA (rRNA) combined with fluorescent in situ hybridization (FISH) (Wagner et al., 1993; 1994a; 1994b; 1995), 16S ribosomal DNA (rDNA) extraction followed by cloning (Snaidr et al., 1997; Layton et al., 2000), or gradient gel electrophoresis. The common attribute of each of these studies was that they involved the successful manipulation of genetic material.

There are several barriers to the manipulation of genetic material including, lack of DNA or RNA isolation or poor yield from isolation protocols, degraded genomic material, and the contamination of genetic samples with enzyme inhibitors (McPherson et al., 1995). Genetic material is usually extracted from bacterial cells either by chemical lysis (Symonds et al., 2009), physical disruption (Layton et al., 2000; Dar et al., 2005; Spano et al., 2005; Park et al., 2006; Siripong et al., 2006) of the cell wall, or a combination of chemical lysis and physical disruption of the cell wall (Lachmayr et al., 2009). Depending on cell wall composition and structure, chemical lysis of bacterial cells may produce a lower DNA or RNA yield. Isolation protocols that use high-speed centrifugation or excessive vortexing may sheer DNA, thus disrupting potential PCR amplification targets. Extraction protocols that do not use filters may allow the coprecipitation of humic compounds along with DNA or RNA. Humic compounds often act as inhibitory compounds to enzymatic processes such as PCR (Atlas and Bartha, 1997).

The purpose of this study was to test seven commercially available genomic DNA isolation kits and methods to determine which would yield genomic DNA that could be easily detected by ethidium bromide gel electrophoresis and amplified by 16S rDNA PCR. The isolation of a genomic DNA product free from humic substances is a likely candidate for PCR amplification. Development or selection of a protocol that provides genomic DNA of sufficient quantity to detect by ethidium bromide staining and gel electrophoresis and is usable for downstream applications such as PCR will allow for the tracking and enumerating of species of interest within the aerobic digest community.

MATERIALS AND METHODS

Cultures

Escherichia coli and Serratia marcescens were used as pure culture controls for each isolation

protocol. Each culture was grown overnight at 37 °C in nutrient broth (8.0 g nutrient broth [Difco] per 1000 ml distilled water). Aerobic digest influent and effluent samples were collected from the Five Mile Creek Wastewater treatment facility in Birmingham, AL. These were maintained at -20 °C until 24 h prior to use; then samples were transferred to 4 °C to thaw completely before use.

Modified Wizard Kit

Genomic DNA was isolated using the Wizard® Genomic DNA Purification kit [Promega]. One ml of each sample was transferred to a sterile 1.5 ml microcentrifuge tube [Fisher] and mixed with 600 µl of Lysis Solution (120 µl 0.5 M EDTA [Promega] and 500 µl Nuclei Lysis Solution [Promega]) and 200 µl Proteinase K (20 mg ml-1 [Fisher]). Samples were incubated at room temperature (21 °C) for 96 h with continuous agitation. After incubation, 3 µl RNase Solution [Promega] was added to each sample and incubated for 1 hr at 37 °C in a dry bath incubator. Samples were cooled to room temperature for 5 min before 200 µl Protein Precipitation Solution [Promega] was added; samples were then mixed by inversion and incubated for 5 min at 4 °C. Proteins were cleared from each sample by centrifugation (4 min at 10 000 rpm [Eppendorf model 5424]). The supernatant was transferred to a sterile microcentrifuge tube, and 600 µl isopropanol [Fisher] was added to each sample. The samples were inverted to mix, and DNA was precipitated overnight at -20 °C. Each sample was split into aliquots of 450 µl sample, and 600 µl isopropanol was again added to samples. The samples were incubated overnight at -20 °C to precipitate DNA. The samples were centrifuged at 12 000 rpm for 2 min at room temperature to collect genomic DNA. The isopropanol supernatant was decanted and discarded, and the DNA pellet was washed in 200 µl 80 % ethanol (80 ml ethanol [Fisher], 200 ml distilled water). The samples were centrifuged at room temperature at 12 000 rpm for 2 min to collect genomic DNA. The supernatant was decanted and discarded, and the genomic DNA pellet was allowed to air dry overnight at room temperature before resuspending in 25 µl DNA rehydration solution (10 mM Tris, 1 mM EDTA [Promega]). Like samples were pooled and stored at -20 °C until used.

Modified Activated Sludge Genomic DNA Extraction Protocol

Genomic DNA was extracted from each sample using the protocol described by Watanabe et al. (1998). One ml of each culture was mixed with 100 µl cell dispersion solution (18.40 g sodium tripolyphosphate [Fisher] per liter DDI H2O) and mixed by vortexing at maximum speed using a Vortex Genie 2 [Fisher]. Dispersed cells were centrifuged for 6 min at 6000 rpm [Eppendorf model 5424] to collect cells. Supernatant solution was carefully decanted and discarded, and the cell pellet was resuspended in 250 µl cell suspending buffer (10 mM Tris HCl pH 8.0 [Fisher], 1 mM EDTA pH 8.0 [Fisher], 0.35 M sucrose [Fisher] per liter DDI H2O) and 250 µl lysozyme (20 mg ml-1 [Fisher]) and incubated at 37 °C for 10 min. To each sample, 375 µl cell lysis solution [Promega] was added and mixed by inversion before incubating for 30 min at 55 °C. Four rounds of phenol-chloroform (24:25 v / v [Fisher, Fisher]) extraction were carried out with the phenol layer being transferred to

a clean 1.5 ml microcentrifuge tube [Fisher] with each extraction. After the fourth round of phenol-chloroform extraction, 500 µl isopropanol [Fisher] was added to each tube and incubated overnight at -20 °C. Nucleic acids were collected by centrifugation at 12 000 rpm for 5 min at room temperature. The supernatant was decanted and discarded, and the nucleic acid pellet was washed in 500 µl 80 % ethanol (80 ml ethanol [Fisher], 20 ml distilled water). The samples were mixed by inversion, incubated for 5 min at room temperature and centrifuged at 12 000 rpm for 2 min at room temperature. The supernatant was decanted and discarded, and the nucleic acid pellet was air dried overnight at room temperature to evaporate any remaining ethanol from the samples. The nucleic acid pellet was resuspended in 250 µl TE buffer (80 mL 100 mM Tris-HCl [Fisher], 8 ml 100 mM EDTA [Fisher] per liter H2O) and incubated at 37 °C for 1 hr before adding 10 µl RNase solution [Promega] to each sample and incubating overnight at 30 °C to degrade RNA. Like samples were pooled and stored at -20 °C until used.

PowerSoil™ DNA Isolation kit (MO BIO Laboratories, Inc.)

One ml of each culture was used to obtain genomic DNA according to the protocol prescribed by the manufacturer. Cultures were added to a Power Bead tube containing 60 µl Solution C1 [MO BIO] and mixed for 10 min using a vortex machine set to high (level 10 on Vortex Genie [Fisher]). The samples were centrifuged for 30 sec at room temperature at 10 000 rpm [Eppendorf model 5424] to collect beads. The supernatant (500 µl) from each sample was transferred to a clean 2 ml microcentrifuge tube [MO BIO], mixed with 250 µl Solution C2 [MO BIO] by vortex for 5 sec at room temperature and incubated 5 min at 4 °C. The samples were centrifuged for 1 min at room temperature at 10 000 rpm. The supernatant (600 µl) was transferred to a clean 2 ml centrifuge tube, and 200 µl Solution C3 [MO BIO] was added to each sample. The samples were mixed by vortex and incubated for 5 min at 4 °C to precipitate proteins. Each sample was centrifuged for 1 min at room temperature at 10 000 rpm to clear the supernatant. For each sample, 700 µl of cleared supernatant was transferred to a new microcentrifuge tube and mixed with 1200 µl Solution C4 [MO BIO] by vortex for 5 sec at room temperature. In aliquots of 675 µl, samples were added to a spin filter and centrifuged at 10 000 rpm for 1 min at room temperature. After centrifugation, the spin filter flow through was discarded. After all samples had been processed, 500 µl Solution C5 [MO BIO] was added to each spin filter and centrifuged for 30 sec at 10 000 rpm. Spin filters were dried by centrifugation for 1 min at 10 000 rpm at room temperature. Spin columns were transferred to a clean 2 ml microcentrifuge tube, and 100 µl Solution C6 [MO BIO] was added to each spin column before centrifuging for 30 sec at 10 000 rpm at room temperature to elute DNA. Like samples were pooled and stored at -20 °C until used.

UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories, Inc.)

Each culture (1.8 ml) was added to a 2 ml collection tube and centrifuged [Eppendorf model 5424] at 12 000 rpm for 30 sec at room temperature to collect bacterial cells or biosolids. The supernatant was decanted and discarded, and the cell pellet was resuspended in 300

µl of MicroBead Solution [MO BIO] before transferring to a MicroBead tube [MO BIO]. Solution MD1 (50 µl [MO BIO]) was added to each sample, and samples were incubated at 70 °C for 10 min to facilitate cell lysis. The samples were vortexed for 10 min at room temperature at high speed (level 10 on Vortex Genie) [Fisher] to physically lyse cells. MicroBeads were collected by centrifugation at room temperature for 30 sec at 12 000 rpm. The supernatant was transferred to a clean 2 ml collection tube, and 100 µl Solution MD2 [MO BIO] was added to each sample and mixed by vortexing for 5 sec at room temperature. Samples were incubated for 5 min at 4 °C to precipitate proteins and centrifuged at 12 000 rpm at room temperature for 1 min to clear proteins. Cleared supernatant (approximately 450 µl) was transferred to a new 2 ml microcentrifuge tube and mixed with 900 µl Solution MD3. Samples were mixed by vortex for 5 sec at room temperature before loading in aliquots of 700 µl onto a spin filter [MO BIO]. Each sample was processed by centrifuging for 30 sec at room temperature at 12 000 rpm with the sample flow through being discarded after each round of centrifugation. After the entire sample had been processed, 300 µl Solution MD4 [MO BIO] was added to each spin filter and centrifuged for 30 sec at 12 000 rpm at room temperature. The flow through was discarded, and the spin filter was dried by centrifugation for 1 min at 12 000 rpm at room temperature. Spin filters were transferred to new 2 ml microcentrifuge tubes, and DNA was eluted from each spin filter using 50 µl Solution MD5 [MO BIO] and centrifuged at 30 sec at 12 000 rpm at room temperature. Like samples of genomic DNA were pooled and stored at -20 °C until used.

UltraClean™ Fecal DNA Kit (MO BIO Laboratories, Inc.)

The samples were processed according to manufacturer's directions. Two hundred-fifty µl (approximately 0.25 g) of each sample was added to a clean 2 ml Fecal Dry Bead tube [MO BIO] and mixed with 550 µl Fecal Bead Solution [MO BIO]. The samples were mixed by gently vortexing at room temperature for 5 sec using a Vortex Genie [Fisher]. Solution S1 (60 µl [MO BIO]) was added to each solution and mixed by inversion. To degrade humic substances, 200 µl Solution IRS was added to each sample, as genomic DNA would be used in PCR. The samples were vortexted at maximum speed (level 10) for 10 min at room temperature to physically disrupt cell walls. Beads were collected after vortexing by centrifuging [Eppendorf model 5424] samples at 12 000 rpm for 30 sec at room temperature. The supernatant was transferred to a clean microcentrifuge tube [MO BIO] and mixed with 250 µl Solution S2 by vortexing for 5 sec at room temperature. The samples were incubated for 5 min at 4 °C to precipitate proteins. Proteins were cleared by centrifugation for 1 min at 12 000 rpm at room temperature. For each sample, 450 µl of cleared supernatant were transferred to a clean microcentrifuge tube and mixed with 900 µl Solution S3 [MO BIO] by vortex for 5 sec at room temperature. Samples were processed in aliquots of 700 µl by loading samples onto spin filters [MO BIO] and centrifuging for 1 min at room temperature at 12 000 rpm. The flow through was discarded. After the final aliquots of samples were processed, 300 µl Solution S4 [MO BIO] was added to each sample and centrifuged for 30 sec at 12 000 rpm at room temperature. The flow through was discarded, and the spin filters were dried by centrifugation for 1 min at 12 000 rpm

at room temperature. The spin filters were transferred to a new 2 ml microcentrifuge tube, and DNA was eluted by adding 50 µl Solution S5 [MO BIO] to each spin filter and centrifuging for 30 sec at room temperature at 12 000 rpm. Like samples of genomic DNA were pooled and stored at -20 °C until used.

UltraClean™ Mega Soil DNA Kit (MO BIO Laboratories, Inc.)

The manufacturer's protocol was modified to process a smaller sample as described below. For each sample, 500 µl culture was added to approximately 500 µl dry beads [MO BIO] and 800 µl Bead Solution [MO BIO]. The samples were vigorously mixed for 1 min using a Vortex Genie [Fisher]. To each sample, 200 µl Solution S1 [MO BIO] was added and mixed by vigorous vortexing for 30 sec at room temperature. Solution IRS (300 µl [MO BIO]) was added to each sample as isolated genomic DNA would be used in PCR amplification. The samples were vortexed on high speed (level 10) at room temperature for 10 min before beads were collected by centrifugation [Eppendorf model 5424] at 12 000 rpm for 30 sec at room temperature. Supernatant was transferred to a clean microcentrifuge tube [MO BIO] and mixed by inversion with 100 µl Solution S2 [MO BIO]. The samples were incubated at 4 °C for 5 min to precipitate proteins. Proteins were cleared by centrifugation for 1 min at 12 000 rpm at room temperature. Cleared supernatant (approximately 500 µl) was transferred to a clean microcentrifuge tube and mixed with 900 µl Solution S3 [MO BIO]. The samples were processed onto a minicolumn [Promega Wizard SV™] in aliquots of 700 µl by centrifugation at 12 000 rpm at room temperature for 30 sec. The minicolumn flow through was discarded. After all aliquots had been processed, 300 µl Solution S4 [MO BIO] was added to each minicolumn and centrifuged at room temperature for 30 sec at 12 000 rpm. The flow though was discarded, and the minicolumn was dried by centrifugation at 12 000 rpm for 1 min at room temperature. The minicolumn was transferred to a clean 1.5 ml microcentrifuge tube [Fisher], and 50 µl Solution S5 [MO BIO] was added to each column to elute genomic DNA by centrifugation at 12 000 rpm for 30 sec at room temperature. Like samples of genomic DNA were pooled and stored at -20 °C until used.

PowerMax™ Soil DNA Isolation Kit (MO BIO Laboratories, Inc.)

The manufacturer's protocol was modified to process a smaller sample as described below. For each sample 500 µl culture, 500 µl dry Power Beads [MO BIO] and 800 µl Power Bead Solution [MO BIO] were added to a 2 ml microcentrifuge tube [Fisher]. The samples were gently vortexed using a Vortex Genie [Fisher] for 1 min at a low setting to thoroughly mix samples. Solution C1 (200 µl, [MO BIO]) was added to samples, and samples were vortexed at the highest level (level 10) for 10 min at room temperature to physically lyse cells. Power Beads were collected by centrifugation [Eppendorf model 5424] at 12 000 rpm for 30 sec at room temperature. The supernatant (approximately 450 µl) was transferred to a clean 1.5 ml microcentrifuge tube [Fisher] and mixed by inversion with 100 µl Solution C2 [MO BIO]. The samples were incubated for 5 min at 4 °C to precipitate proteins before clearing proteins by centrifugation for 1 min at 12 000 rpm at room temperature. The supernatant (400 µl) was transferred to a clean 1.5 ml microcentrifuge tube and mixed

by inversion with 300 µl Solution C4 [MO BIO]. The samples were processed onto a minicolumn [Promega Wizard SV™] in aliquots of 700 µl and centrifuged at 12 000 rpm for 30 sec at room temperature. The minicolumn flow through was discarded after each round of centrifugation. After the last aliquot was processed for each sample, 100 µl Solution C5 [MO BIO] was added to minicolumns and centrifuged at 12 000 rpm for 30 sec at room temperature. The minicolumns were dried by centrifugation at 12 000 rpm for 1 min at room temperature. The minicolumns were transferred to a clean 1.5 ml microcentrifuge tube. To elute genomic DNA, 50 µl Solution C6 [MO BIO] was added to each minicolumn and centrifuged at 12 000 rpm for 30 sec at room temperature. Like samples were pooled and stored at -20 °C until used.

Detection of Genomic DNA

To determine the presence of genomic DNA from each extraction method, 15 µl of genomic DNA was mixed with 3 µl 6 X loading dye solution (10 mM Tris-HCl pH 7.6, 10 mM EDTA, 0.005 % bromophenol blue, 0.005 % xylene cyanol FF, and 10 % glycerol [Fermentas]). Eighteen µl of each sample was run on a 0.8 % agarose [Fisher] gel in 1 X TAE buffer (40 mM Tris-acetate, 1 mM EDTA pH 8.3 [Sigma]) for 45 min at 120 V in an electrophoresis chamber [Fisher] at room temperature. Gels were stained in 0.5 µg ml-1 ethidium bromide [MO BIO] staining solution (ethidium bromide in distilled water) for 30 min at room temperature. Gels were visualized using a UV light box [Fisher]. The presence of bands indicated that genomic DNA was of sufficient concentration to be detected by gel electrophoresis and ethidium bromide staining.

Amplification of Genomic DNA

A primer pair specific to the 16S rDNA region of domain bacteria was used to amplify a 1.5 Kb band for aerobic digest influent, aerobic digest effluent, and E. coli genomic DNA samples. Amplification was carried out according to the following reaction: 12.5 µl 2 X GoTaq® Green Master Mix (GoTaq® DNA Polymerase, 2X Green GoTaq® Reaction Buffer pH 8.5, 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM MgCl2 [Promega]), 0.625 µl E8F (Baker et al., 2003) forward primer (sequence: 5' AGAGTTTGATCATGGCTCAG 3', 5 pmol [Integrated DNA Technologies]), 0.625 µl 1492R reverse primer (sequence: 5'GGTTACCTTGTTACGACTT 3', 5 pmol [Integrated DNA Technologies]), 11.0 µl nuclease free H2O [Promega], and 0.25 µl template DNA (diluted 1:5 with nuclease free H2O or undiluted) to make a final volume of 25.0 µl. A water negative and Pseudomonas aeruginosa genomic DNA sample were amplified to ensure that reagents were not contaminated and that PCR was successful. The P. aeruginosa reaction used the same reaction and primer pair as reported for the aerobic digest influent, aerobic digest effluent, and E. coli samples. The PCR reaction was amplified in an Eppendorf Mastercycler gradient thermocycler at 94 °C for 5 min to allow for initial denaturation of genomic DNA; then, 45 cycles were performed at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1.5 min, followed by a final extension time of 8 min at 72 °C. Samples (20 µl) were viewed on a 0.8 % agarose gel in 1X TAE buffer containing ethidium bromide as previously described.

RESULTS

Genomic DNA was isolated from influent and effluent aerobic digester samples using Promega's Wizard® Genomic DNA Purification kit, MO BIO Laboratories' UltraClean™ Microbial DNA Isolation kit, UltraClean™ Fecal DNA kit, and UltraClean™ Mega Soil DNA kit and detected by gel electrophoresis and ethidium bromide staining (Table 1). Genomic DNA was also successfully isolated from E. coli with each of these kits. However, due to low cell density of the overnight culture, genomic DNA isolated from S. marcescens was of insufficient yield to be detected by gel electrophoresis and ethidium bromide staining. The UltraClean™ Mega Soil kit gave the sharpest genomic band with the least amount of shearing as determined by visual analysis of all protocols tested.

Genomic DNA was not detectable by gel electrophoresis and ethidium bromide staining in either aerobic digest samples or pure culture samples when extractions were performed using a modified version of the activated sludge genomic DNA extraction protocol (Watanabe et al., 1998), MO BIO Laboratories' PowerSoil™ DNA Isolation kit, or MO BIO Laboratories' PowerMax™ Soil DNA Isolation kit (Table 1). It is unclear if this is due to poor bacterial cell lysis or low DNA recovery.

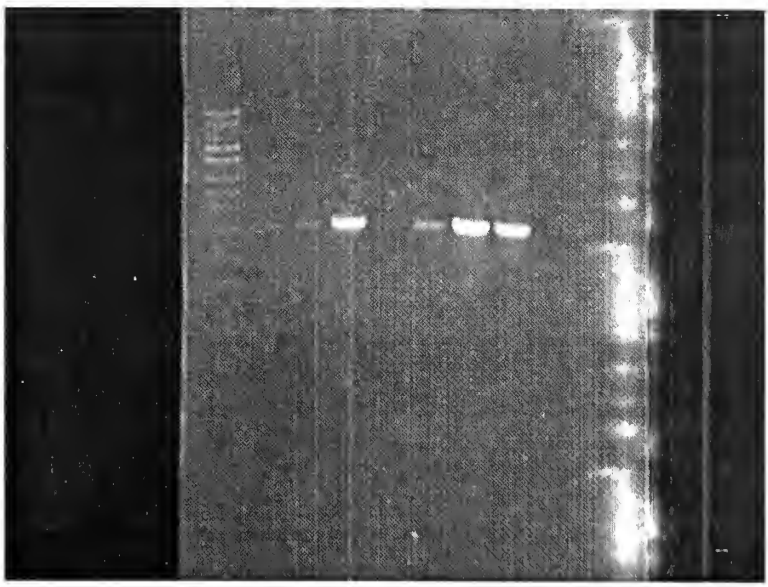

Figure 1: Amplification of UltraClean ™ Fecal DNA samples using universal primers E8F and 1492R. Samples are (1) 1 KB exACTGene DNA marker [Fisher], (2) aerobic digest influent (diluted 1:5), (3) aerobic digestion effluent (diluted 1:5), (4) E. coli (diluted 1:5), (5) aerobic digest influent (undiluted), (6) aerobic digest effluent (undiluted), (7) E. coli (undiluted), (8) positive control P. aeruginosa (diluted 1:10), and (8) negative control (H2O).

Promega's Wizard® Genomic DNA Purification kit produced a DNA product from the aerobic digest samples that was amber in color (Table 1). The amber pigmentation of these samples suggests that a humic acid is present. Extraction procedures that used a spin filter or minicolumn successfully removed the amber co-precipitate from the genomic DNA samples. No amber pigment was observed with pure culture samples (e.g., E. coli).

A 1.5 Kb band was successfully amplified using genomic DNA samples isolated with the UltraClean™ Fecal DNA kit and GoTaq® Green DNA polymerase (Figure 1). Genomic DNA was amplified using 1:5 dilution of aerobic digest influent (lane 2), 1:5 dilution of aerobic digest effluent (lane 3), 1:5 dilution of E. coli (lane 4), undiluted aerobic digest effluent (lane 6), undiluted E. coli (lane 7), and P. aeruginosa (lane 8). No amplification was observed in the negative (H2O) control or the undiluted aerobic digest influent (lane 5). Due to the small amount of template DNA used (0.25 µl) in the GoTaq® Green amplification protocol, it is possible that no DNA or insufficient DNA was transferred to the undiluted aerobic digest influent sample, thus resulting in no amplification. Unsuccessful amplification reactions used exACTGene Taq polymerase [Fisher] and amplification cycles of 30 or 40 under the same PCR parameters as described above (data not shown). Genomic DNA isolated with the Wizard® Genomic DNA Purification kit, the UltraClean™ Microbial kit, and the UltraClean™ Mega Soil kit did not produce a PCR product when amplified using the GoTaq® Green amplification protocol (data not shown).

DISCUSSION

Several genomic DNA extraction methods were compared to determine which gave a genomic DNA product that was detectable by gel electrophoresis and ethidium bromide staining and was free from humic contaminants. Four commercially available kits successfully extracted genomic DNA that was detectable and contained prominent genomic DNA bands. Three of these kits, the UltraClean™ Microbial DNA Isolation kit, the UltraClean™ Fecal DNA kit, and the UltraClean™ Mega Soil DNA kit yielded a genomic DNA product that was free from amber pigmentation. One kit, Promega's Wizard® Genomic DNA Purification kit, yielded genomic DNA containing an amber pigment in aerobic digest samples. The amber pigmentation suggests the presence of a humic substance, a known inhibitor to downstream applications such as PCR (Atlas and Bartha, 1997). Preliminary experiments using amber pigmented genomic DNA extracted from aerobic digest samples have not been successful in PCR amplification of 16S rDNA (data not shown).

Seven genomic DNA kits and methods were tested; the UltraClean™ Mega Soil DNA kit gave the sharpest genomic DNA band with the least amount of shearing. However, the UltraClean™ Mega Soil DNA kit is designed to process large soil samples, and the kit must be modified to use with smaller sample volumes. This kit is rather expensive per prep compared with the UltraClean™ Fecal DNA kit, which also yields a genomic

DNA product within a short amount of time and has little shearing of the genomic DNA. Samples extracted with the. UltraClean™ Fecal DNA kit are capable of being amplified by PCR. The UltraClean™ Microbial DNA kit is also cost effective and yields DNA within a short amount of time, but genomic DNA is greatly sheared and could not be amplified by PCR. Promega's Wizard® Genomic DNA Purification kit requires several days to extract genomic DNA, and the DNA is contaminated with humic acid. The UltraClean™ Mega Soil DNA kit, Fecal DNA kit, and Microbial DNA kit are all suitable for rapid extraction of genomic DNA from complex samples. All are easy to use, therefore making them suitable for routine use in the research laboratory as well as the teaching laboratory. As no genomic DNA was isolated with the modified activated sludge genomic DNA extraction protocol suggested by Watanabe et al. (1998), the PowerSoil™ DNA Isolation kit, or the PowerMax™ Soil DNA Isolation kit, these methods are not recommended for isolation of genomic DNA from complex microbial communities

Both aerobic digest influent and effluent samples extracted by the UltraClean™ Fecal DNA kit produced a 1.5 Kb band by PCR amplification with universal primers E8F and 1492R. E. coli samples were included as an extraction controls. Pure cultures are expected to be free from organic contaminants such as humic acids that inhibit PCR. E. coli is often used to test for fecal contamination of water and is known to be present in aerobic digest samples collected at the Five Mile Creek Wastewater Treatment facility (data not shown). The detection of E. coli shows that further amplification reactions can be developed to detect pathogens or specific organisms of interest in wastewater.

Table 1. Summary of genomic DNA extraction protocols as determined by gel electrophoresis of genomic DNA samples and stained with ethidium bromide (EtBr).

Extraction Method	Shearing	Band Sharpness	Pigmentation	Detected By EtBr	Cost per Prep	Ease of Use	PCR Amplification	Overall Rating
Wizard kit	None observed	Smear	Yes- sludge samples only	Yes	$1.35	Moderate	No	4
Activated Sludge	None observed	None observed	None observed	Not detected	NA	Difficult	Not tested	5
PowerSoil Isolation kit	None observed	None observed	None observed	Not detected	$4.16	Easy	Not tested	5
UltraClean Microbial kit	Yes	Yes, with smear	None observed	Yes	$2.00	Easy	No	3
UltraClean Fecal kit	Yes	Yes, little smearing	None observed	Yes	$3.88	Easy	Yes	1
UltraClean Mega Soil kit	None observed	Yes	None observed	Yes	$17.80	Easy	Not tested	2
PowerMax Soil kit	None observed	None observed	None observed	Not detected	$18.90	Easy	Not tested	5

Obtaining a genomic DNA product that can be successfully amplified using PCR or other molecular methods will allow for a more in-depth study of the microbial community of aerobic digest. Understanding this community offers several benefits: pathogens may be monitored for long-term survival, problematic species that cause poor dewatering of sludge or sludge bulking and foaming may be detected earlier thus avoiding treatment problems, and the effect of treatment plant operations can more

rapidly be determined and modified. Through the use of culture independent methods, monitoring of species of interest is no longer limited to organisms that can be cultured using traditional agars, nor will species that have long generation times be problematic as molecular methods provide results within hours of sample collection and processing.

Techniques used with genomic DNA isolation from aerobic digest may also be applied to other complex microbial communities such as soils and sediments that may generate samples contaminated with humic acids.

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MOVEMENT OF MERCURY FROM A CONTAMINATED CITY PARK IN OXFORD INTO THE COOSA RIVER, ALABAMA

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ABSTRACT

Soil samples from an Oxford, AL, city park and from sites within the same watershed but outside the park area were collected and analyzed for mercury (Hg) content using cold vapor atomic absorption. Soil from the park had a mean Hg concentration of 3.27 ± 0.59 µg/g (mean ± SEM., n = 12). Mean Hg concentration for soil collected outside of the park was 0.04 ± 0.01 µg/g (n = 12). Sediment samples were collected from Snow Creek as it flows through the Oxford park and from Snow Creek upstream of the park. When analyzed for Hg, sediment from the park had a mean concentration of 1.21 ± 0.13 µg/g (n = 19), while sediment collected upstream of the park had a concentration of 0.12 ± 0.03 µg/g (n = 19). A profile of Hg concentrations in sediments collected from Snow Creek and Choccolocco Creek downstream of the park showed areas with higher Hg concentrations extending 36 miles to the Coosa River. Earthworms (*Lumbricus terrestris*) grown in soil from the Oxford park had significantly higher tissue levels of Hg than earthworms grown in background soil. The data support the hypothesis that Hg from Oxford park soil is being transported downstream from Snow Creek to the Coosa River.

INTRODUCTION

We have identified a city park, Oxford Lake Park, in Oxford, AL, where the concentration of Hg in the soil is significantly higher than that found in soil samples collected from areas surrounding the park (Figure. 1). We propose that fill deposits used as landscape materials in the park were contaminated with mercury (Hg) from an industrial source. It is not clear from public records when this landscaping occurred, although it was prior to 2003. A likely source for this fill material was an industrial chlor-alkali facility where a mercury cell process had been used to produce chlorine gas for the manufacture of chlorinated biphenyls. The mercury cell was operational from 1952 until 1969 (Bluemilk, 2001). Unlike organic contaminants which generally undergo decomposition over time due to natural oxidation or microbial metabolism, heavy metals are in the elemental state and cannot be further broken down. Once released into the environment, heavy metals such as Hg can constitute a long-lasting hazard. Although these metals are not degraded, they can be changed from one oxidation state to another depending on local conditions

(Schwedt, 2001). Such changes affect both the mobility and the bioavailability of metals. Mercury in the environment can occur as elemental mercury (Hg^0), ionic mercury (Hg_2^{2+}, Hg^{2+}) or alkylated mercury (CH_3Hg^+ or $(CH_3)_2Hg$). These forms can coexist and often interchange as a result of interactions with organic material (Si and Ariya, 2008), bacteria (Lefebvre et al., 2007), or flora (Leonard et al., 1998). Such changes can alter not only the water solubility and bioavailability of Hg, but also the volatility.

The oxidation state of Hg has a pronounced effect on its toxicity in humans. Inhaled elemental Hg vapor readily penetrates the lungs, and can distribute from the blood into the central nervous system (CNS) (Hamada and Osame, 1996). Mercury accumulation in the CNS produces a syndrome characterized by tremors, excitability, insomnia, and depression. Ionic Hg is toxic to the kidney, causing a form of glomerulonephritis (Liu et al., 2008). Most human exposure to methyl Hg results from the consumption of fish. Almost all of the Hg in most fish species occurs as the alkylated form, CH_3Hg^+. Methyl Hg readily crosses lipid barriers in the body, including the blood-brain barrier and the placenta. In the CNS, methyl Hg can cause cerebral edema, gliosis, and cerebral atrophy. In children exposed prenatally, methyl mercury can produce a form of cerebral palsy (Liu et al., 2008).

Snow Creek, a third order perennial stream, flows through Oxford Lake Park. It is relatively calm with alternating pools and riffles. Snow Creek joins Choccolocco Creek approximately one mile downstream from the park. Choccolocco Creek empties into Logan-Martin Reservoir on the Coosa River. We previously reported that fish collected from Snow Creek as it passes through Oxford Lake Park had significantly higher tissue levels of Hg than fish collected from Snow Creek upstream from the park (Kohute et al., 2006). The July 26, 2008, edition of the *Anniston Star* published a fish advisory for consumption of spotted bass from Choccolocco Creek due to high levels of Hg (Faulk, 2008). This advisory applies to a reach of Choccolocco Creek downstream from the confluence with Snow Creek. In the present study, data is presented that supports the hypothesis that soil from Oxford Lake Park is a source of the Hg found in the Choccolocco Creek fish.

MATERIALS AND METHODS

Soil and Sediment Preparation

Twenty-eight grab samples of the Snow and Choccolocco Creek's bottom sediments were taken at convenient points averaging no more than 1.5-mile apart along a 36-mile longitudinal traverse from its headwater to its confluence with Logan Martin Reservoir (Figure 1). These grab sediment samples from the creek bottom were retrieved by a non-metal trowel and placed in a sterile 120 ml glass jar with a Teflon-lined cap.

Between 4.65 and 7.57 miles along the transverse, a spike in Hg level was detected (Figure 4). At the location of this spike in Hg level, biased soil samples were grabbed from the floodplain of the creek. These samples were taken less than 10 feet from the creek bank. Three grab samples of soil, which were from the upper 12 cm of soil, were mixed thoroughly and placed in a sterile 120 ml clear glass jar with a Teflon-lined cap. To avoid

cross contamination, the Teflon spade used to extract soil was brushed clean after each sample. Any grass or rock was separated from each field sample.

Vertical profiles of soil in the same location as the biased samples and in the adjacent bottom of Snow Creek were extracted by a 30 cm long AMS Slotted Soil Recovery Probe with a 2.2 cm diameter butyrate liner. The recovered profile was sealed with plastic end caps and wrapped in aluminum foil for transportation and storage.

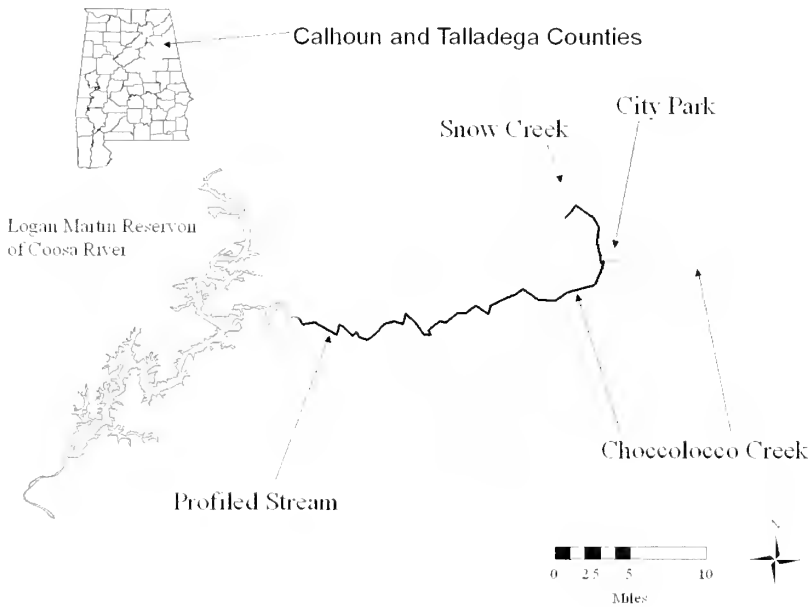

Figure 1. Location map indicating Snow and Choccolocco Creeks and the City Park.

For determination of total concentration of Hg in the soil, field samples were divided into triplicate analytical samples, each weighing 2.000 g (± 0.001 g). Each two gram analytical sample was placed in an acid washed 500 mL BOD bottle and partially dissolved in 50 mL ultra-pure water (17 megaohm resistance). Mercury in the samples was oxidized to Hg^{2+} using 0.5 mL trace metal grade sulfuric acid, 0.25 mL trace metal grade nitric acid, 1.5 mL potassium permanganate solution (5% wt/wt), and 0.8 mL of potassium persulfate solution (5% wt/wt). Samples were then heated at 95 °C for two hours. Samples were allowed to cool to room temperature. Immediately before analysis, 1.0 mL of hydroxylamine solution (12% wt/wt) was added to each sample to reduce unreacted potassium permanganate. The solution phase from each BOD bottle was transferred to an analyzer tube by syringe with a sediment filter attachment. Sediment samples were collected and processed in a similar manner.

Earthworm Treatment and Preparation

Earthworms, *Lumbricus terrestris*, were obtained from DMF Bait Company (Waterford, MI). Due to a two-year severe local drought during the time of this research, we were not able to collect earthworms from local soils. Worms were housed in 1.38×10^{-2} m^3 styrofoam boxes filled with either contaminated soil collected from the Oxford park or background soil collected from a local field. The background soil had a Hg concentration of 0.03 ± 0.02 µg/g (mean ± S.D., n = 3). Worms were added to the boxes on day one. After two weeks, ten worms were taken from each box for Hg analysis. Worms ranged in size from 10-20 cm at the time of harvesting. Worms were also harvested for analysis at four-week and six-week time intervals. After harvesting, worms were measured and euthanized by cooling. Worms were frozen at -70 °C for a minimum of 24 h and then freeze-dried (VirTis Freezemobile 12) for 3-4 days. Freeze-dried tissue was pulverized with a mortar and pestle. Triplicate 2.00 g samples were taken from each batch of worms and digested with a mixture of 15 mL of trace metal grade nitric acid and 2.0 ml of 30% hydrogen peroxide (Fisher OPTIMA grade). Digested tissue was dissolved in 7% trace metal grade nitric acid and filtered (Fisherbrand Q8 filter paper) into 50 ml volumetric flasks. The mixture was diluted to 50 mL with 7% nitric acid. Prior to analysis, samples were further digested in sulfuric acid, nitric acid, potassium persulfate, and potassium permanganate by heating to 95 °C for one h. This treatment insured that all forms of Hg were converted to Hg^{2+}. Immediately before analysis, 1 mL of 12% hydroxylamine (wt/wt) was added to each sample.

To determine the fraction of Hg contained in the worms that could be attributed to soil in the worm's gastrointestinal (GI) tract, five worms were randomly selected and carefully dissected to remove the intact GI tract. For each worm, the GI tract (and the soil it contained) was separated from the remaining tissue, and both were freeze-dried. Masses of the resulting dried materials were determined to three decimal places.

Mercury Analysis

All reagents used in sample preparation and Hg analysis were trace metal grade. Ultra-pure water (17 megaohm resistance) was used to prepare all solutions. Standard Hg solutions were prepared from a Fisher Certified Mercury Reference Solution, Lot No. 055614-24. All glassware was acid washed prior to each assay. Samples were analyzed for total Hg using USEPA Method 245.1, Manual Cold Vapor Technique (USEPA, 1983). Mercury analysis was conducted using a CETAC Quick Trace Mercury Analyzer M-6100 cold vapor atomic absorption Hg analyzer with an ASX-400 AutoSampler. All specimens were run in batches that included blanks (reagent and instrument), a five point standard calibration curve (standards of 0.0, 0.5, 1.0, 2.5, 5.0, and 10.0 µg/L with a linear correlation of 0.995 or better), and spiked specimens. Matrix spikes gave 85-90% recovery. Specimen split between two batches had a variation of less than 5%. The calculated method detection limit (MDL) for Hg in the liquid phase was 0.1 µg/L. NIST Standard Reference Material 2976, mussel tissue, was used to validate the analytical procedure. The concentration of Hg in the reference material was reported as 0.061 µg/g. The experimental value determined in this study was 0.058 µg/g.

Statistical Analyses

GraphPad Prism, version 5.01 (GraphPad Software, Inc., San Diego, CA), was used for data analyses. The unpaired t test was performed to identify differences between contaminated groups and background groups. A one-way ANOVA was used to compare Hg uptake over time for earthworms. Results were considered significant at an α level of .05. Means are reported ± SEM.

RESULTS

In this study, evidence is presented that soil from Oxford Lake Park has significantly higher Hg levels than soil collected from sites within the same watershed but outside the contaminated park area. In addition, sediment collected from Snow Creek as it flows through the park is shown to have significantly higher Hg levels than sediment collected from Snow Creek upstream of the park. A profile of Hg concentrations in Snow Creek sediments extending from the park downstream into Choccolocco Creek, and continuing downstream for a distance of 36 mile to the Coosa River is presented. Earthworms, *Lumbricus terrestris*, grown in soil from the park are shown to accumulate significantly higher tissue concentrations of Hg compared to earthworms grown in soil having the area background concentration of Hg. Earthworms have been reported to accumulate Hg when grown in Hg contaminated soils (Ernst and Frey, 2007). As earthworms are a food source for many animals, they could serve as a mechanism by which Hg from soil enters the terrestrial food chain.

Mercury in Park Soil

We analyzed 12 grab samples collected from the upper 12 cm of soil at 7 sites locations in the floodplain of Oxford Lake Park located adjacent to the area of Snow Creek that produced the spike observed in the bottom sediment (Figure 4). These data were compared to 12 grab soil samples analyzed from 12 sites from the Snow Creek Watershed outside of the contaminated area of the park. Soil samples collected from the park had a mean Hg concentration of 3.27 ± 0.59 µg/g whereas background soil samples had a mean of 0.04 ± 0.01 µg/g (Figure 2). Soil samples collected from the park had significantly higher mean Hg levels than soil collected outside of the park but in the same flood plain ($t = 5.448$, $P = <0.0001$).

Mercury in Creek Sediment

Nineteen bottom sediment grab samples were collected from Snow Creek as it flows through Oxford Park and nineteen grab sediment samples were collected from Snow Creek upstream of the park. Park bottom sediment samples had a mean Hg concentration of 1.21 ± 0.13 µg/g. Bottom sediment samples collected from Snow Creek upstream of the park had a mean Hg concentration of 0.12 ± 0.03 µg/g (Figure 3). Mercury levels were significantly higher in sediment from the park compared to upstream ($t = 8.048$, $P = <0.0001$).

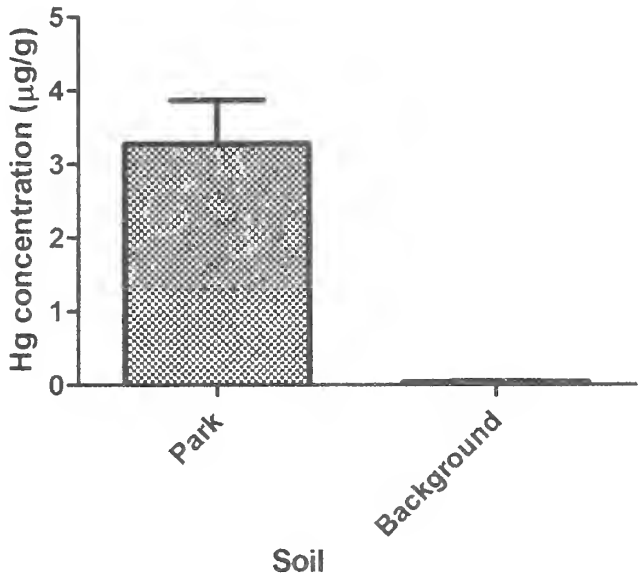

Figure 2. Mercury concentrations in park and background soils.

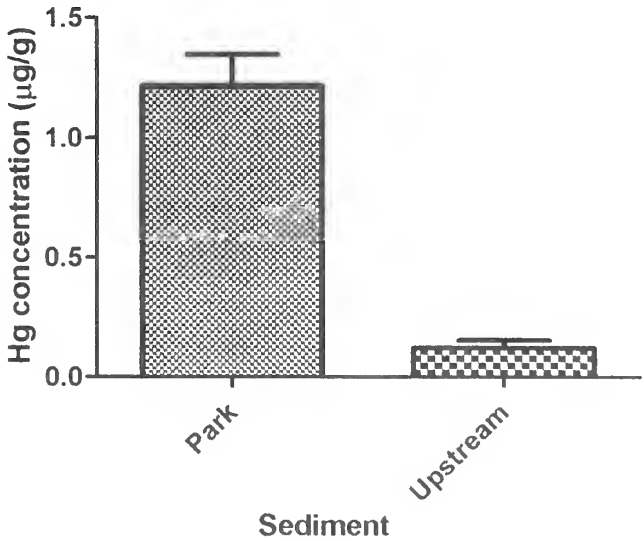

Figure 3. Mercury concentrations in park and upstream sediments. Distribution of Hg contaminated sediment.

Figure 4 depicts the longitudinal profile of Hg levels in the bottom sediments of Snow Creek continuing downstream into Choccolocco Creek. Each point represents the mean of three replicate samples. These are biased samples collected where mud had collected on the stream bed. The profile starts (0 mile) at an unnamed tributary of Snow Creek upstream of the Oxford park and ends 36 miles downstream at the outflow point of Choccolocco Creek into Logan Martin Reservoir on the Coosa River (Figure 1—darkened stream segments). A spike in Hg concentrations occurs between 4.65 and 7.57 miles along the profile. This corresponds to the area where Snow Creek passes through the Oxford Lake Park and an adjacent shopping mall. Downstream from the park, Snow Creek feeds into Choccolocco Creek which outflows into Logan Martin Reservoir on the Coosa River.

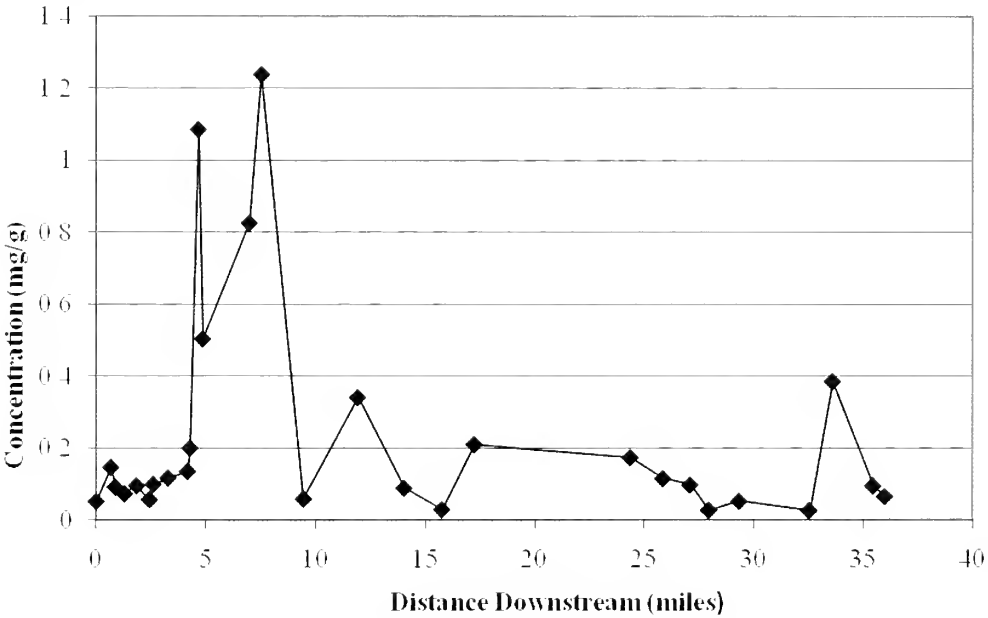

Figure 4. Sediment concentrations of Hg in Snow and Choccolocco Creeks.

Vertical Profiles of Hg Distribution

Vertical profiles were determined for Hg distribution in both the contaminated soil of the park and the contaminated creek bottom sediment in the park (Figure 5). Each point represents the mean of three replicate samples. Both the park soil and the creek sediment show some level of vertical variation of Hg levels. These profiles suggest that the eroded contaminated soil becomes more homogenized when it is redistributed in the stream bottom sediment load. The measurement of the vertical extent of the contamination both in the soil and sediment was restricted by the corer used to extract the soil/sediment profiles.

Mercury Uptake by Earthworms

In an earlier study (McLaughlin et al., 2007), we presented evidence that earthworms were bioaccumulating Hg from the contaminated soil in the park. This bioaccumulation could be an important mechanism in the vertical and horizontal re-distribution of Hg in soil. Due to the extended drought in the study area, naturally occurring earthworms were hard to sample. Consequently, a laboratory study of earthworm bioaccumulation was undertaken. Earthworms grown in a sample of background soil with a Hg level of 0.03 µg/g had a total Hg tissue concentration of 0.05 ± 0.004 µg/g (n = 9) dry weight (Figure 6). Earthworms grown in a sample of contaminated park soil having a Hg concentration of 0.52 µg/g had a total Hg tissue concentration of 0.27 ± 0.07 (n = 9). Total tissue Hg levels were significantly higher in worms grown in park soil compared to worms grown in background soil (t = 3.049, P = 0.0077). While small increases in the Hg levels in worms were seen with increased exposure times, these differences were not found to be significant based on one-way ANOVA analysis.

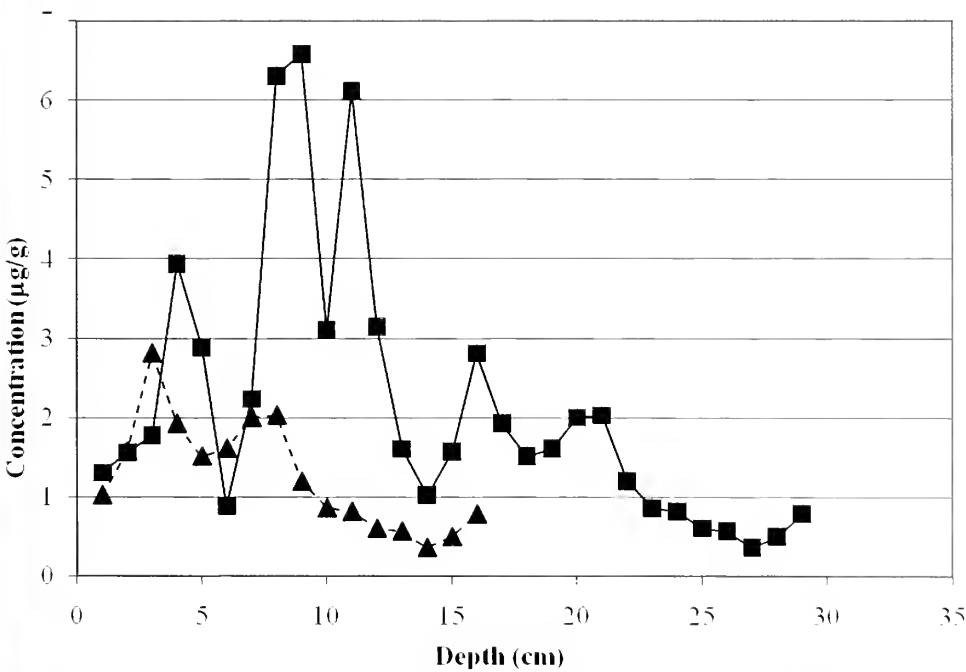

Figure 5. Mercury concentrations at various depths for park soil and stream sediment.

Table 1. Hg concentration in worm tissue and GI tract.

Worm	Total Dry Mass (g)	GI Tract Dry Mass (g)	Tissue Mass % (GI Tract Mass/Total Mass)	Total Hg (mg)	GI Tract Hg (mg)	Hg Mass % (GI Tract Hg/Total Hg)
1	0.639	0.129	20.19	0.173	0.067	38.73
2	0.782	0.122	15.60	0.211	0.063	29.86
3	0.660	0.100	15.15	0.178	0.052	29.21
4	0687	0.093	13.54	0.185	0.048	25.95
5	0.892	0.118	13.23	0.241	0.061	25.31
Mean	0.732	0.112	15.54	0.195	0.058	29.81

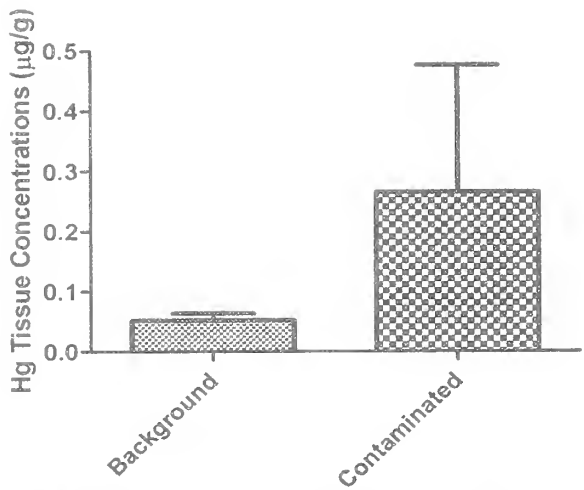

Figure 6. Mercury concentrations in earthworm tissues.

Data presented in Table 1 was used to determine the fraction of Hg detected in the worm that could be attributed to soil contained the worm's GI tract. The first column of Table 1 gives the mean dry mass of five randomly selected worms as 0.732 ± 0.047 g (mean ± SEM). The second column shows the mean dry mass of the five GI tracts plus the soil contained therein to be 0.112 ± 0.007 g. Based on this data, the GI tract plus soil accounted for approximately 16% of the total worm mass (column 3). Worms grown in park soil had a mean Hg tissue concentration of 0.27 µg/g. Using this tissue concentration to calculate the total body Hg concentration, the five worms in this study contain a mean of 0.195 ±

0.012 µg of Hg per worm (Table 1). The park soil had a Hg concentration of 0.52 µg/g. Assuming that the entire mass of the GI tract in each worm was soil, this calculates to a mean of 0.058 ± 0.004 µg of Hg per GI tract. Thus, the Hg in the GI tract of a worm should only account for 30-35% of the Hg total in a worm.

DISCUSSION

The association of environmental Hg contamination with chlor-alkali facilities is well documented (Santschi et al., 1999; Zagury et al., 2006; Neculita et al., 2005). An article in the Anniston newspaper (Bluemink, 2001) chronicles Hg release into the local environment by a chlor-alkali facility that operated from 1952 until 1969. This article reports a 1970 advisory for Hg in fish from Choccoloccо Creek downstream from this facility. While there are public accounts of Hg releases from the former chlor-alkali facility, there does not seem to be any public information explaining the use of soil contaminated with Hg as landfill at Oxford Lake Park. However, there is a public record indicating that the company that operated the chlor-alkali facility was involved in the development of the park. There are also public accounts that both Oxford Lake Park and Snow Creek as it flows through the park had been contaminated with chlorinated biphenyls (PCBs) (Dougan, 2000; USEPA, 2001). The Dougan report attributes PCB contamination of park soil to past flooding of Snow Creek. We propose that the Hg contamination and probably the PCB contamination (although we have yet to investigated this) are the result of contaminated soil use as fill deposits in the park. Our data show significantly higher Hg levels in soil collected from Oxford Lake Park compared to soil samples collected from the same drainage system outside of the contaminated park area. If Hg in the park soil resulted from the flooding of Snow Creek, it would be expected that flood prone areas upstream from the park would also have higher than background Hg levels. This was not found to be the case. Mercury levels in the sediment of Snow Creek as it flows through the park were significantly higher than Hg levels in sediment collected from Snow Creek upstream of the park. This argues against the downstream movement of Hg as the source of the current Hg contamination in park soil. Additional support of the premise that Hg is entering Snow Creek from the park soil and not from an upstream source comes from a previous study in which we found that fish collected from the park area of Snow Creek had significantly higher tissue Hg levels than fish of the same species collected from Snow Creek upstream of the park (Kohute, et al., 2006).

Our data support the hypothesis that Hg from the park soil is moving into Snow Creek and being transported to Choccoloccо Creek and downstream into the Coosa River. While it is not clear from the public records how the soil in Oxford Lake Park became contaminated with Hg, our data indicate that the Hg is not remaining sequestered in the park soil. Mercury is moving into Snow Creek where it has entered the aquatic food chain. Mercury from park soil may be entering the terrestrial food chain due to uptake by earthworms. Earthworms grown in contaminated soil from the park had Hg tissue levels over five times higher than earthworms grown in background soil. Atmospheric deposition

of Hg from coal burning power plants is a source of Hg contamination across northern Alabama (Nichols et al., 2002), and could account for the background level of Hg seen in our study. However, atmospheric deposition does not explain the high Hg level found solely in the park.

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SAFEGUARDING REVENUE-GENERATING ASSETS IN AUSTERE ECONOMIC ENVIRONMENTS: A CASE STUDY OF DADEVILLE, ALABAMA

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ABSTRACT

Across the United States, many states and local communities have invested in or otherwise facilitated tourist and recreational development. While these types of investments have increasingly contributed to 'green' development, just as importantly, these investments in leisure activities have proven to be significant economic assets that enhance a region's potential for residential, commercial, and industrial growth. Of particular note is the state of Alabama's Robert Trent Jones Golf Trail, which spans the entire state and includes eleven trail sites with twenty-six golf courses. As part of the Retirement Systems of Alabama's (RSA's) investments, the primary objective of the golf trail is to effectively diversify the state's asset base and strengthen its overall economy. As of 2006, this statewide recreational activity has contributed nearly two billion dollars to the RSA pension fund. In a similar way, although on a smaller scale, Lake Martin serves a number of surrounding local communities and counties with several positive economic and recreational opportunities. Presently, however, these benefits are at risk because of poorly monitored and unmanaged wastewater discharges flowing into Lake Martin. Within this context, this study documents the environmental degradation and attendant economic loss associated with unmanaged wastewater discharges into Lake Martin.

INTRODUCTION

Across the United States, many state and local governments directly invest public funds into recreational/tourism 'infrastructure' and/or devise public programs that facilitate and attract private sector investment into recreational/tourism activities (Cromartie 2001; Johnson and Beale, 2002; Reeder and Brown, 2005). While these types of activities are relevant and timely in today's society, especially as they positively contribute to 'green' and sustainable planning, equally important is that these same activities often serve as economic catalysts, possessing the potential to attract in additional economic growth and development. In particular, thoughtfully planned recreational/tourism activities may significantly increase the attractiveness (i.e., residential, commercial, and industrial) of a community, or state. The planning concepts of national and state parks, second home

developments with secluded, lake vistas, and commercial/light industry districts serving casinos or amusement parks are all exemplary of economic boosters for local and state government coffers. Of note, however, is that the significance and sustainability of these and other economic boosters depend on the core activity with which they are connected—recreation/tourism. The criticality of this connection is especially problematic for smaller, semi-rural communities that inherently possess limited economic resources and alternative capabilities. Specifically, if a potentially productive recreational/tourism activity's actual or perceived attractiveness declines, then its economic potential to attract additional growth and development declines commensurately. Within this context, this study examines Lake Martin, located primarily in Tallapoosa County, Alabama, as a recreational/tourism activity that potentially provides superior economic growth and development benefits not only for Tallapoosa and other surrounding counties as a whole but also for several local communities (e.g., Dadeville and Alexander City) in particular. Presently, Lake Martin provides the city of Dadeville with a plethora of economic growth and recreational-based opportunities including but not limited to primary and second home development, ancillary commercial enterprises, and water sporting events for tourists. However, the community of Dadeville, along with Tallapoosa County, is at risk of losing these potential economic benefits. Poorly monitored and unmanaged wastewater discharges from Dadeville's wastewater treatment plant are flowing into Lake Martin at toxic levels. Furthermore, political and civic efforts to safeguard Lake Martin have been virtually blocked and ineffective.

Throughout the past twenty-five years, recreation/tourism has been a growing industry (Alabama Department of Economic and Community Affairs, 2002; Travel Industry Association of America, 2004; U.S. Department of Commerce, 2007). In 2006, recreation/tourism generated over one trillion dollars and employed 8.5 million people across the United States. Specifically, for the state of Alabama, recreation/tourism attracted over 22 million visitors who contributed over 8.3 billion dollars to the state's economy and generated nearly 650 million dollars in tax revenue that was shared between the state and local governments (Alabama Bureau of Tourism and Travel, 2006). With such significant total revenue associated with recreation/tourism in the state, it is imperative for both state and local governments to develop and preserve the growth of this industry. Of particular note is that nature-based recreation/tourism represents a significant part of this industry in the state of Alabama. Communities that possess well- developed nature-based opportunities are highly likely to attract not only a diversity of commercial enterprises, but relocating retirees. Kerr (1991) states: "Unlike new companies that often demand millions of dollars in tax incentives for setting up shop in an area, retirees and their hundreds of thousands of dollars in assets can be drawn in with a brochure, a tour, a handshake, and a smile." According to the U.S. Bureau of Census (2005) more than one-half of relocating retirees in 2004 reported incomes exceeding sixty thousand dollars, and twenty-five percent reported incomes over one hundred thousand dollars.

Presently, there are two recreational/tourism activities in particular that provide both the state of Alabama and several of its local communities with substantial economic and social benefits—the Robert Trent Jones (RTJ) Golf Trail and the Windy Creek State Park.

The RTJ Golf Trail includes several championship caliber golf courses designed by Robert Trent Jones, Sr. and geographically dispersed across the state. The Trail includes eleven sites with twenty-six golf courses that were designed to aesthetically blend in with their surrounding natural landscapes. The Trail concept, developed by David Bronner, CEO of the Retirement Systems of Alabama, was created to diversify the assets of the state's pension fund and strengthen the overall economy of the state. To date, the success of the RTJ Golf Trail has contributed nearly two billion dollars to the RSA's pension fund. In addition to this sizable economic benefit to the state of Alabama, the Golf Trail has been a catalyst for attracting substantial residential and commercial development into the respective local communities. For example, seven world-class resorts and spas, along with a plethora of retail/commercial businesses, have located in seven of the eleven Golf Trail sites, Table 1. Moreover, nearly all of the Golf Trail sites have residential development occurring coterminous with the golf courses, with a median housing price exceeding one-half million dollars.

Table 1. Listing of the Robert Trent Jones Golf Trails by location and accompanying spa/resort development.

Golf Course	City	County	Spa/Resort Development
Cambrian Ridge	Greenville	Butler	n.a.
Capital Hill	Prattville	Autauga	Montgomery Marriott Hotel and Conference Center; Renaissance Montgomery Hotel and Spa
Grand National	Opelika	Lee	Auburn Marriott Opelika Hotel and Conference Center
Hampton Cove	Huntsville	Madison	n.a.
Highland Oak	Dothan	Houston	n.a.
Lakewood Golf Club	Point Clear	Mobile	Grand Hotel Marriott Point Clear Resort and Spa
Magnolia Grove	Mobile	Mobile	Battle House Renaissance Hotel; Renaissance Riverview Plaza Hotel
Oxmoor Valley	Birmingham	Jefferson	n.a.
Ross Bridge	Hoover	Jefferson	Renaissance Ross Bridge Golf Resort and Spa
The Shoals	Muscle Shoals	Colbert	Marriott Shoals Hotel and Spa
Silver Lakes	Anniston/ Gadsden	Calhoun/ Etowah	n.a.

A second significant recreation/tourism example in the state of Alabama is the Windy Creek State Park (which includes Lake Martin). The state park is located below Alexander City in Tallapoosa County and spans nearly 1,500 acres of pine and hardwood ridges along Lake Martin's shoreline. Offering over six hundred campsites, a fully developed marina, pavilions, nature trails, and a fishing pier, the park's main attraction is Lake Martin. Lake Martin is one of Alabama's largest manmade lakes and provides nearly year-round water sporting events including competitive sport fishing. Annually, over fifty thousand dollars in revenue is generated through park use and directly distributed to the local government of Alexander City, monies that are earmarked to fund

the city's Gateway to Education Scholarship Assistance Program. Importantly, both of the aforementioned examples demonstrate how nature-based recreation/tourism serves as a significant economic resource to state and especially local communities. And because these activities depend on local community facilities to support and safeguard their development, it is vital that state, county, and city governments ensure that local community facilities are properly regulated, monitored and managed. Within this context, this study examines toxic levels of wastewater discharge flowing into Lake Martin (via the city of Dadeville's wastewater treatment plant) and the potential negative impact on Lake Martin to continue to serve as the recreational centerpiece of Windy Creek State Park.

CASE STUDY

Tallapoosa County was created by the Alabama State Legislature in 1832; the city of Dadeville, which serves as the county seat for Tallapoosa County, was surveyed in 1836 and granted a charter in 1837. Not until 1923, when the Martin dam was constructed, was Lake Martin created. At the time of its completion, Lake Martin was the world's largest manmade lake, spanning forty-four thousand acres across three counties with over seven hundred miles of shoreline. With a predominantly rural-based economy, both Tallapoosa County and the city of Dadeville depend heavily on primary sector activities. As a result, both of these political entities have experienced a population loss of younger residents (specifically 15-34 years), out-migrating to other more industrialized counties across the state. At the same time, however, and in contrast to national and state statistics, Tallapoosa County and the city of Dadeville have experienced a continuous in-migration of people aged 40 and older, Figure 1. Between 1997 and 2002, Tallapoosa County lost seven manufacturing establishments, totaling over one billion dollars in revenue and 2,600 jobs. Further evidence of this area's prevailing rural-based economy with limited alternatives is that in the year 2000: (1) only fifty-seven percent of Tallapoosa County's population sixteen years of age and older constituted its labor force, and only ninety-four percent of the labor force was gainfully employed; (2) per capita income was significantly lower than the U.S. average; and (3) nearly twenty percent of the region's population earned a household income below the national poverty level, Figures 2 and 3.

In contrast, since 1990 the Lake Martin area has experienced dramatic growth in the recreational use of the lake and, in turn, has precipitated substantial development of

retirement residential communities along with ancillary services (Arnberg, 2007). For example, in 2006, the local tourism and travel industry: (1) generated over five percent of the state of Alabama's total gross domestic product, (2) experienced a twenty-nine percent increase in direct travel-related employment—totaling 688 new jobs, (3) increased total travel-related earnings by thirty-four percent—exceeding fourteen million dollars in revenue, and (4) accounted for nearly sixty percent of all fishing expenditures in the state of Alabama. Moreover, in 2001, Tallapoosa County appraised at $2.3 billion; however, by 2006, the appraised value of the county had increased nearly thirty-three percent to $3.2 billion. To a large extent, this increase was directly attributable to increased residential construction associated with Lake Martin. Specifically, a one billion dollar residential community, The Ridge, is currently under construction that will include up to 1,500 housing units with projected selling prices ranging from 500,000 to 900,000 thousand dollars.

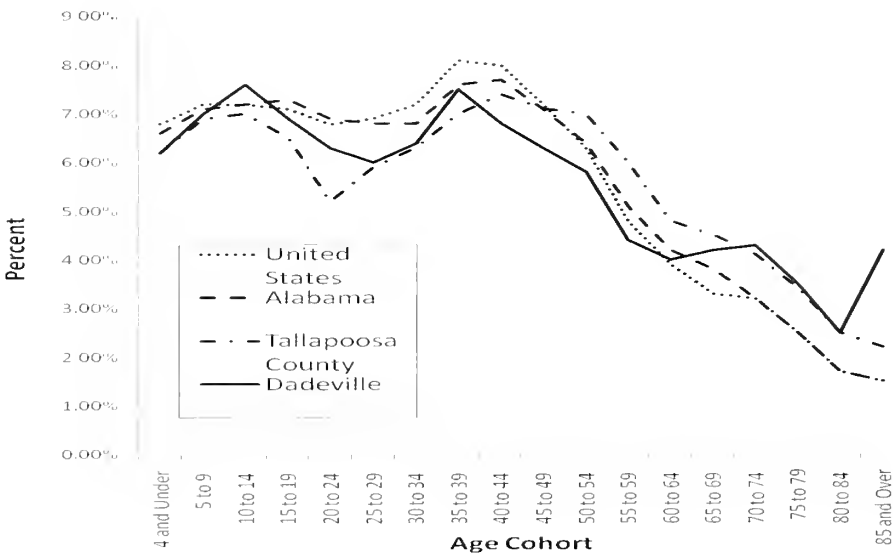

Figure 1. Percent of age group in population for the United States, Alabama, Tallapoosa County, and the city of Dadeville, 2000.

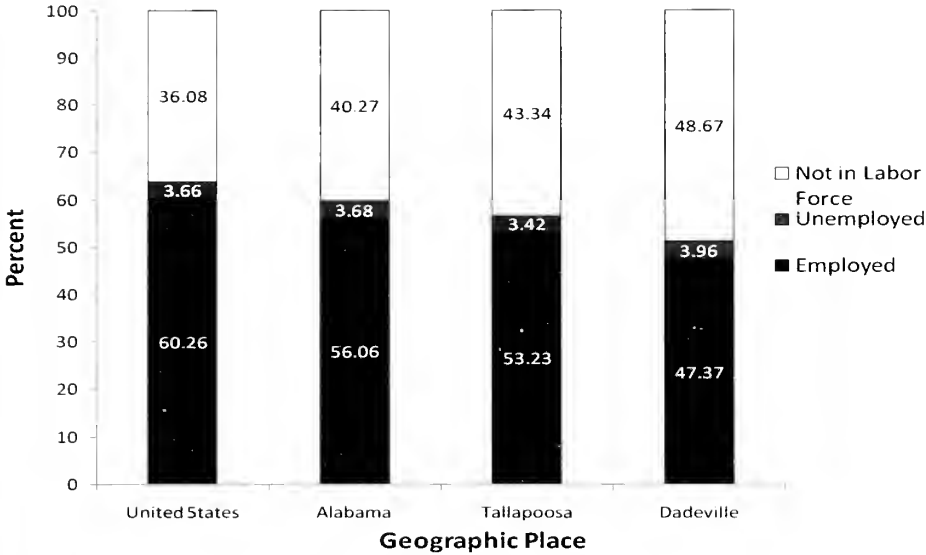

Figure 2. Employment status in percent of popualtion 16+ years old for the United States, Alabama, Tallapoosa County, and the city of Dadeville, 2000.

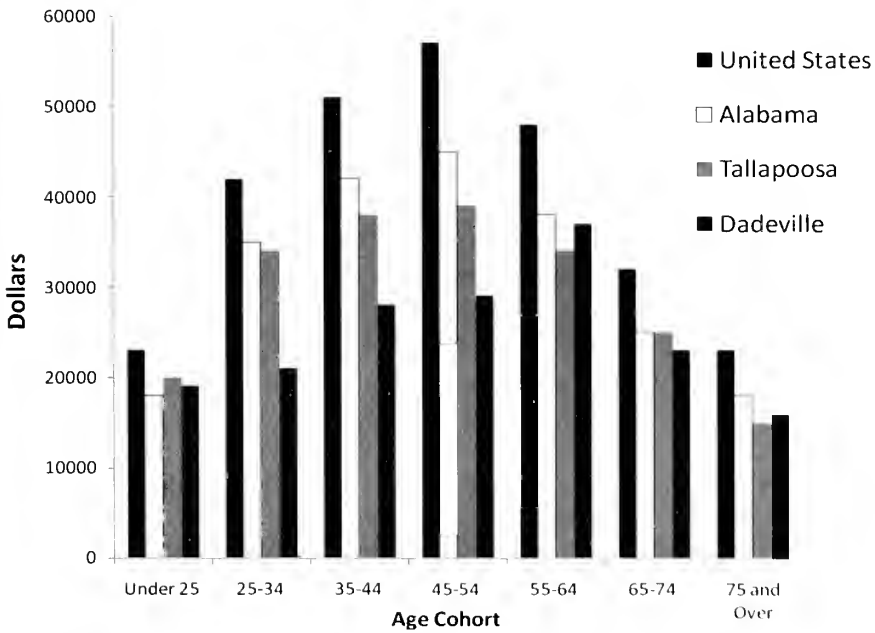

Figure 3. Median household income by age for the United States, Alabama, Tallapoosa County, and the city of Dadeville, 1999.

Since the 1990s, baby boomers have increasingly considered rental and ownership of second home properties in rural environments that possess attractive natural and cultural amenities. In fact, rural communities that were once predominantly seasonal have increasingly become occupied with a growing retiree population that is remaining year-round. Importantly, as retirees decide to permanently move into an area, they stimulate and diversify the local economy by attracting more seasonal vacationers, bolstering the local economy, and encouraging local government to improve local services. Over time as population and local market demand increase, new businesses are attracted to the area, creating new jobs and expanding the local economy. In turn, poverty levels decrease, land values appreciate, health care services expand, and more tax revenue is generated. This time-driven process, however, may never culminate in overall economic improvement, especially if the initial triggering mechanism (e.g., Lake Martin) becomes compromised or diminished in its strategic economic value.

The City of Dadeville's Impact

Over the past fifteen years, there has been a well documented and growing concern related to the city of Dadeville's wastewater flowing into Lake Martin. This is of critical concern, especially to the counties and local communities served by the lake insofar that Lake Martin functions as both a significant economic contributor to their economies as well their primary source for drinking water. If the lake becomes unsuitable for both human and economic use, the negative impact (e.g., health, residential, and economic) on the Dadeville area, Tallapoosa County, as well the neighboring counties and communities will be significant.

Regulatory Background

In 1977, an amendment to the Federal Water Pollution Control Act (1972), the Clean Water Act, defined the basic structure for regulating discharges of pollutants into bodies of water in the United States. This law empowered the U.S. Environmental Protection Agency (EPA) to set effluent standards on an industry-by-industry basis and established water quality standards for all contaminants in surface waters. By 1987, the Clean Water Act was reauthorized and expanded to recognize more chemical pollutants, authorize citizen suit provisions, and fund publically-owned treatment works that enabled local communities to construct wastewater treatment facilities. In addition, the 1987 Act allowed the EPA to delegate permitting, administrative, and enforcement aspects of the law to state governments. In the state of Alabama, these delegated aspects of the Clean Water Act fall under the purview of the Alabama Department of Environmental Management (ADEM). In addition, The Safe Drinking Water Act (1974) also applies to the city of Dadeville's discharging its wastewater into Lake Martin since the lake is a primary source for the surrounding region's drinking water. Under this act, the EPA not only establishes drinking water standards to monitor and control the level of toxic contaminants but also conducts an oversight review of the standards every five years.

Dadeville's Wastewater Treatment Facility

The city of Dadeville's wastewater treatment facility is nearly thirty years old, and

according to the Clean Watersheds Needs Survey (2000), the facility had a receiving population of nearly two thousand people and a daily flow of 355,000 gallons of wastewater with a maximum operating flow capacity of 425,000 gallons. At the time the survey was conducted, Dadeville's treatment facility was already operating at eighty-five percent capacity with a forecasted receiving population of four thousand people and no plans to increase its operating flow. Importantly, if the facility were to reach full capacity, any additional incoming wastewater would be diverted untreated into a nearby local stream, Chattasofka Creek. Of critical importance is that Chattasofka Creek flows into Sandy Creek, which empties into the residential and recreational areas of Lake Martin.

Between 2002-2007, a total of fifty-eight notices of violation and sanitary sewer overflow (SSO) events issued to the city of Dadeville were on file with ADEM, Table 2. The more significant of these violations are associated with high levels of fecal coli form, suspended solids, ammonia nitrate, residual chlorine, dissolved oxygen, and pH. In fact, over eighty-five percent of all the violations cited involve excessive permissible levels of fecal coli form and ammonia nitrate. Both of these contaminants pose serious potential negative health and bio-aquatic effects. Escherichia coli (E. coli) are the most recognizable member of the coli form group and may, along with the presence of related pathogenic bacteria, make Lake Martin an unsafe source for drinking water. Moreover, long-term excessive levels of nitrates and nitrites may cause hemorrhaging in a human's spleen. Finally, ammonia nitrate is also a nutrient that stimulates lake eutrophication, causing pervasive algae growth that could significantly impact the lake's wildlife and especially its fish population.

Table 2. A list of fifty-eight notice of violations and SSO events issued to the city of Dadeville, 2002-2007.

Year	Notice of Violations/SSO Events
2002	NH_4NO_3(6), Cl(5), SS(1), FCw(1), FCs(3)
2003	NH4NO3(2), FCw(4), FCs(4)
2004	NH_4NO_3(6), FCw(4), FCs(2)
2005	FCw(3), FCs(1), SSO(4)
2006	NH_4NO_3(4), FCw(1), FCs(2), SSO(1)
2007	NH_4NO_3(3), FCw(1)

NH_4NO_3: Ammonia Nitrate, Cl: Chlorine, SS: Suspended Solid, FCw: Fecal Coliforms (winter), FCs: Fecal Coliforms (summer), SSO: Sanitary Sewer Overflow

In 2005, the ADEM issued a notice of violation to the city of Dadeville to correct the ammonia nitrate violations that had occurred nearly one year earlier. Subsequently, a second notice of violation was issued in 2006, forcing Dadeville to recognize not only its excessive levels of coli form and ammonia nitrate but also its increasing infiltration and inflow problem. Specifically, Dadeville's wastewater treatment facility was discharging untreated water into public waterways, an SSO event. Under ADEM regulations,

wastewater treatment facilities are required to record and notify ADEM verbally (within 24 hours of the event) and in writing (within five days of the event). In less than one year (April 2005 to February 2006), Dadeville's wastewater treatment facility incurred five SSO events, and subsequently five additional SSO events, occurred in 2007, none of which were reported to ADEM. If Lake Martin continues to be increasingly exposed to these contaminants, the surrounding communities and counties will likely experience not only significant human health issues but also a constriction in their local economies. For these reasons, careful monitoring and regulation of Dadeville's wastewater treatment facility remains a compelling issue.

In early 2007, residents and some local businesses partnered to form grass roots organizations including Lake Watch and Lake Martin Home/Boat Owners Association in an effort to address the problem of untreated water being discharged into both Sandy Creek and Lake Martin. The organizations retained legal counsel and filed a sixty-day notice of intent to sue the city of Dadeville for their continued inaction to safeguard Lake Martin against excessive contaminant levels from influent toxins. This notice of intent elicited two immediate legal responses, one from ADEM and a second from the Attorney General of Alabama. First, a special order of consent was issued from ADEM to Dadeville that included a monetary penalty and a list of specific requirements to correct the water discharge problem from its wastewater treatment facility. After Dadeville's mayor signed the special order of consent, public notice of the consent order was published in local newspapers throughout the region. A formal public hearing was held resulting in written assurances from ADEM that careful monitoring and corrective measures would be required of the city of Dadeville to rectify all water discharge problems with its wastewater treatment facility Second, and immediately following ADEM's action, Alabama's attorney general filed a civil complaint against the city of Dadeville that focused on a variety of water quality permit violations. In early spring 2008, the city of Dadeville responded by denying all allegations and demanded strict proof otherwise. No follow-up action has yet been taken by the state.

CONCLUSION

When the City of Dadeville applied to renew the permit for its wastewater treatment facility, Lake Watch member sent a letter to the director of ADEM specifically requesting that the permit be renewed for only two years, as opposed to the customary five-year period and furthermore suggested that the city of Dadeville publish a monthly summary of its discharge monitoring report in the local newspapers. On July 6, 2007, ADEM renewed Dadeville's waste water treatment plant permit without mention or consideration of the documented illegal discharges. Employing Frazer Lanier financing company, Dadeville's city council approved a one million dollar bond issue to finance the plant's upgrade from an operating capacity of 425,000 gallons/day to 750,000 gallons/day and to make all necessary repairs to sewer lines to prevent rain water from infiltrating the sewer system.

Although there appears to be new hope not only for the residents of Sandy Creek and Lake Martin but also the local communities impacted by the tourist dollars generated by

Lake Martin, the future of this economic asset remains uncertain, especially if policymakers continue to mismanage and poorly monitor potentially toxic environmental events. For example, a recent study in New Hampshire determined that the economic cost associated with a decline in the state's recreational water resource amounted to nearly fifty-one million dollars in reduced total sales, eighteen million dollars less in household income, and a loss of over eight hundred jobs (Colburn and Teutsch, 2007). Therefore, if local officials can both recognize and quantitatively measure the economic benefits and costs associated with preserving and monitoring Lake Martin as a significant revenue-generating resource, then any resources necessary for corrective action and continuous safeguard monitoring should be developed expeditiously. Further research would include answering questions including: (1) what are the long term goals for the Lake Martin area and how might the contaminants negatively impact these goals? (2) are the numbers and/or size of specific forms of aquatic life being impacted? (3) are fish safe for human consumption, or is there a limit on quantity that may be consumed? and (4) what is the potential impact on shoreline vegetation, leading to erosion and property value depreciation?

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BROMINATION OF DIMETHYL MALEATE USING BROMOFORM AS CATALYST UNDER DIFFERENT ENERGY SOURCES: A CASE STUDY FOR ITS ROLE IN BIOTRANSFORMATIONS

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ABSTRACT

Maleic acid (cis-2-butene-1, 4-dioic acid, Lide, 2004) and its corresponding methyl ester, dimethyl maleate (DMME) in which the alkene double bond is in conjugation with a carboxyl group, undergo isomerization on treatment with catalytic amounts of aqueous bromine or Br_2/CCl_4 under UV light to form the thermodynamically more stable trans isomer. There are limited reports on the possible formation of any addition products, product selectivity or stereochemistry as affected by the light or heat source. In order to optimize the conditions for a more selective, stereospecific addition or isomerization, and to better understand the similar processes in biological systems, reactions were performed treating dimethyl maleate (DMME) with bromine using different energy sources such as heat, sunlight, and a UV lamp (wavelength, 365 nm) with varied solvent concentration. On treatment with slight excess of bromine (1:1.1 equivalent), the reaction gave the addition product selectively when heated to reflux in CCl_4 (bp 77°C). Whereas reaction with a catalytic amount of bromine or bromoform in CCl_4, isomerization occurred. Treatment of DMME with excess bromoform under UV irradiation for 4-5 days gave selectively the isomerized product in 95 % yield via bromine released as an intermediate. The reaction mechanism, product selectivity, stereochemistry, and the role of such processes in the biological systems are discussed.

INTRODUCTION

While maleic acid, the cis isomer, is a synthetic organic compound, its trans isomer, fumaric acid, is an organic acid widely found in nature (Felthhouse *et al.*, 2001). It is found in plants (*Fumaria Officinalis*), in humans and in other mammals. It is a key intermediate in the citric acid cycle. Both isomers are useful as building blocks for the synthesis of many organic chiral and achiral compounds (Bradshaw *et al.*, 1969). Maleic acid is highly useful as an intermediate in the industrial preparations of polyester resins, plasticizers, copolymers, and agricultural chemicals (Culbertson, 1987). Fumaric acid is used by cells to produce energy from food. Human skin naturally produces fumaric acid when exposed

to sunlight. It is used as a food acidulant and also as an intermediate in the synthesis of certain polyester resins, quick-setting inks, furniture lacquers, paper sizing chemicals and aspartic acid. Recent studies reveal that dimethyl fumarate (DMFE) can be used to treat psoriasis (Schmidta *et al.*, 2007), but there are risk factors involved upon ingestion of this compound. Reactivity of dimethyl fumarate towards glutathione in the preparation of S-substituted thiosuccinic acid esters and its presystemic metabolism has been reported (Langguth *et al*, 2003).

Bromoform is produced naturally in small amounts by oceanic plants and then readily evaporates into the air due to its high volatility (Goodwin *et al.*, 1997). Most of the bromoform that enters the environment is formed as byproduct when chlorine is added to water to kill bacteria (Richardson *et al,* 2003). Halogenated compounds are also released into the air from the use of commercial products (Bao *et al.*, 1998) or the use of haloforms as solvents and reagents in research laboratories and industries. The toxicity of these compounds is associated with their biotransformation (Anders and Jacobson, 1985). Glutathione-dependent biotransformation of vicinal-dihaloalkanes to alkenes and its stereochemistry have been reported earlier (Livesey *et al.*, 1982 and Bao *et al.*, 1998). Organic reactions and mechanistic studies form the basis for the understanding of such biotransformations. This bioactivation can be due to the formation of stable, toxic metabolites or the reactive electrophylic or radical intermediates. Our studies on the reactions of alkene derivatives with bromine and bromoform could form the basis for studies of similar processes in biological systems.

Maleic acid and fumaric acid can not normally be interconverted because rotation around the carbon-carbon double bond is restricted. In the laboratory, the conversion of cis isomer into trans isomer (Pasto *et al*, 1992) is performed by treating maleic acid with aqueous bromine under uv light, and in industry it is produced by catalytic isomerization with mineral acids (Vogel, 1980). Alkenes in general are also known to undergo addition reactions at lower temperatures. Addition of bromine to simple alkenes (Kuwayama *et al.*, 2002) in CCl_4 solvent forms the corresponding dibromoalkane derivatives at room temperature without any added catalyst. Since this reaction introduces two chiral carbons into the product, the cis alkenes are known to form a racemic mixture, and the trans isomer forms a stereospecific meso diastereomer. According to the literature (Mathai *et al.*, 1956), addition of bromine to maleic acid in chloroform solvent occurs in the dark at room temperature but takes several days. There are limited reports (Kajigaeshi *et al.*, 1988) on the effect of any catalysts or other factors such as reaction temperature, solvent or concentration.

In an attempt to achieve the product selectivity between addition and isomerization, and to better understand the reaction mechanism and stereochemistry, and its role in understanding similar processes in the environment and biological systems, a systematic study on the bromination of DMME using halogenated solvent and reagent was undertaken. Our attempts gave interesting and promising results that are discussed herein.

Materials and Methods

Reactions were performed under different conditions by varying the energy sources, temperature, molar concentration, and reagents/catalysts, and were monitored by color change and TLC. For TLC, Merck KGaA flexible plates of 200 micron thickness coated with silica gel, 60Å, F_{254} were used. Products were analyzed and identified by melting point determination and spectroscopic methods. Observed melting points were compared with reported values (Lide, 2004) for all known compounds. IR spectra were recorded on Thermo Scientific, Nicolet 6700 FTIR instrument. NMR spectral analysis was performed on Bruker 300 MHz Instrument. Mass spectrum was recorded on MALDI-TOF mass spectrometer. All spectral data were compared with literature data (Erickson, 1965; Velichko, 1980). The following chemicals and materials were used: DMME (Aldrich reagent + grade, 96 %; 4 % fumarate), $CHBr_3$ (Aldrich, 99+ %), CCl_4 (Fischer, ACS, 99.996 %), Br_2 (Aldrich, reagent + grade), UV lamp (wavelength, 365 nm). UV Reactions (Table 1):

These reactions were conducted in 50 mL conical flasks. The solutions were prepared by taking 20 or 2 mL CCl_4 then adding 0.13 mL of DMME followed by 0.1 mL of $CHBr_3$. The reaction flasks were placed on a magnetic stirrer and under a UV lamp as an energy source. Then, stirring was started. While stirring, 0.1 mL of Br_2 was added drop wise which

Table 1. Reactions under UV (100 watt, 365 nm wavelength) and results

Substrate	Reagent/ Catalyst	Solvent Amount	Molar concn	[b]Reaction Time	% yield	
					Isomerized Product	Addition Product
[a]DMME	-	CCl_4 20 mL	.05 M	1-48 hr	trace	-
[a]DMME	Br_2 (cat; ~0.1 eq)	CCl_4 20 mL	.05 M	0.5 hr	28 %	-
[a]DMME	Br_2 (1 eq)/ $CHBr_3$(cat; ~ 0.1 eq)	CCl_4 2 mL	.5 M	1 hr	-	13 % -
[a]DMME	Br_2 (1eq) No $CHBr_3$	CCl_4 2 mL	.5 M	1 hr	-	26 %
[a]DMME	Br_2 (1 eq)/ $CHBr_3$ (cat; ~ 0.1 eq)	CCl_4 20 mL	.05 M	1 hr	-	43 %
[a]DMME	Br_2 (1eq) No $CHBr_3$	CCl_4 20 mL	.05 M	1 hr	-	76 %
[a]DMME	1 eq $CHBr_3$ No Br_2	CCl_4 20 mL	.05 M	24 hrs	11.2 %	-
[a]DMME	3 eq $CHBr_3$ No Br_2	CCl_4 20 mL	.05 M	24-48 hrs 72-96 hrs	33.7 % 95 %	-

[a]Dimethyl Maleate; [b]Reaction time was determined based on color change from dark scarlet red to light orange or yellow.

gave the solution a scarlet red color. The flasks were left under the UV lamp until the color of the solutions changed from dark red to light orange or yellow (approximately 1hr). After 45 min to 1 hr, the solutions were removed from under the UV lamp, and stirring was stopped. The color change of the reaction mixture from deep red to colorless represented the end point of reaction which was confirmed by TLC analysis. When the reaction was over, and the solution had cooled, white crystals formed. The crystals were vacuum filtered, dried, and checked for melting point and percent yield. The product was further characterized by IR and NMR spectral analysis. Reactions with bromoform in the absence of bromine gave different results, as shown in Table 1.

Sunlight Reactions (Table 2): These reactions were done exactly as the UV reactions except that sunlight was substituted for the UV lamp. The solutions were prepared as described under UV reactions. After adding bromine, the reaction flasks were kept under direct sunlight until the deep red bromine color disappeared. The reaction time and results are given in Table 2.

Table 2. Reactions under sunlight and results

Substrate	Reagent/ Catalyst	Solvent Amount	Rxn Concn.	[b]Reaction Time	% yield	
					Isomerized product	Addition product
[a]DMME	Br_2 (cat; ~ 0.1 eq)	CCl_4 20 mL	.05 M	0.5 hr	45 %	-
[a]DMME	Br_2 (1eq)/ $CHBr_3$ (cat; ~ 0.1 eq)	CCl_4 2 mL	.5 M	1 hr	-	8%
[a]DMME	Br_2 (1eq)/ No $CHBr_3$	CCl_4 2 mL	.5 M	1 hr	-	34% -
[a]DMME	Br_2 (1eq)/ $CHBr_3$ (cat; ~ 0.1 eq)	CCl_4 20 mL	.05 M	1 hr	-	35%
[a]DMME	Br_2 (1eq)/ No $CHBr_3$	CCl_4 20 mL	.05 M	1 hr	-	66%

[a]Dimethyl Maleate; [b]Reaction time was determined based on color change from dark scarlet red to light orange or yellow.

reactions, except the solutions were heated to reflux at the boiling point of the solvent used, using a heating mantle as a heat source. Solutions were prepared by taking 20 mL of CCl4 and then adding 0.13 of DMME. Then Br2 was added to one of the solutions (in this solution CHBr3 was not added), and in the other solution CHBr3 was added without adding the Br2. The reason for this was to see if the Br that is being added is coming from the Br2 or the CHBr3, and also to see if CHBr3 is a better catalyst either for addition or for isomerization to take place. The reactions were continued under reflux conditions for an hour or so, and then they were removed from the heat and left overnight under the hood to cool for the crystals to form. The product crystals were vacuum filtered and left to dry

under the hood for another 24 hours. Afterwards, the melting point and the mass of the crystals were measured. The results and data are given in Tables 1, 2 and 3.

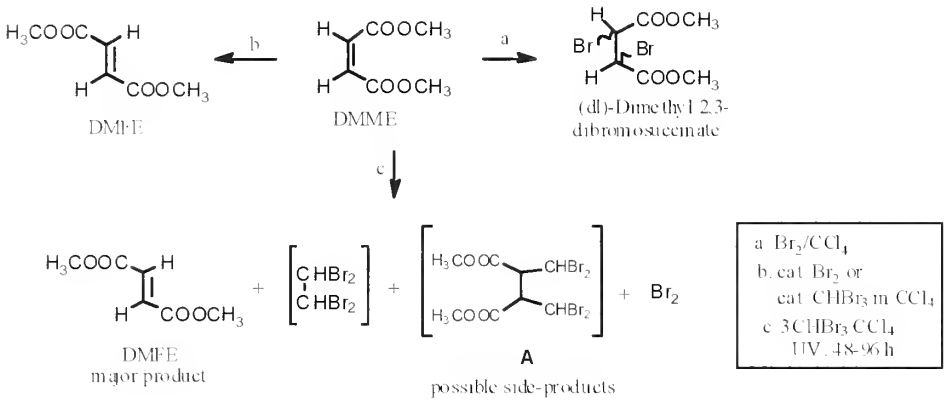
RESULTS AND DISCUSSION

Addition of Bromine to DMME in 1:1 ratio using CCl4 as solvent with 0.05 M concentration under reflux conditions gave the addition product in ~95 % yield with maximum purity (Table 3). Although the yield was lower under sunlight (66 %, Table 2) and UV (76 %, Table1), the reaction process seemed to be faster than under thermal heat source. The addition product was isolated as pure white, needle-like crystals by simply evaporating the solvent at room temperature. The observed mp range for the pure crystals matched the literature value of 57-58oC (Lide, 2004) for the addition product. The IR spectrum showed characteristic saturated ester carbonyl absorption at 1755 cm-1. The product structure was further confirmed by 1H NMR (Fig 1). A 13C- NMR (Fig 2) of the product was also performed and confirmed it to be (d,l)-2,3-dibromosuccinate. All other reactions gave the addition product but in low yield (20 – 40 %) along with trace amounts of the isomerized trans-product. We were unsuccessful in isolating the isomerized product in this set of reactions. The reactions performed are shown in Scheme 1 as a general reaction scheme with the reactants and products.

Table 3. Reactions under Thermal Heat source (reflux at the BP of solvent) and results

Subtrate	Reagent/ Catalyst	Solvent Amount	Rxn Concen.	[b]Rxn Time	% yield	
					Isomerised product	Addition product
[a]DMME Rxn1	Br_2 (cat; 0.1 eq)	CCl_4 20 mL	.05 M	4	58 %	-
[a]DMME Rxn2	Br_2 (1 eq)/ $CHBr_3$ (cat, ~ 0.1 eq)	CCl_4 2 mL	0.5 M	3	-	48 %
[a]DMME Rxn3	Br_2 (1 eq)/ $CHBr_3$((cat, ~ 0.1 eq)	CCl_4 20 mL	0.05 M	6	-	95 %
[a]DMME Rxn4	Br_2 (1 eq) No $CHBr_3$	CCl_4 20 mL	0.05 M	3-4	-	98 %
[a]DMME Rxn5	$CHBr_3$ (cat; ~ 0.1 eq) No Br_2	CCl_4 20 mL	0.05 M	3-4	-	No Reaction-
[a]DMME Rxn6	$CHBr_3$(1 eq) No Br_2	CCl_4 20 mL	0.05 M	24	No reaction	-

[a]Dimethyl Maleate; [b]Reaction time was determined based on color change from dark scarlet red to light orange or yellow.



Scheme 1

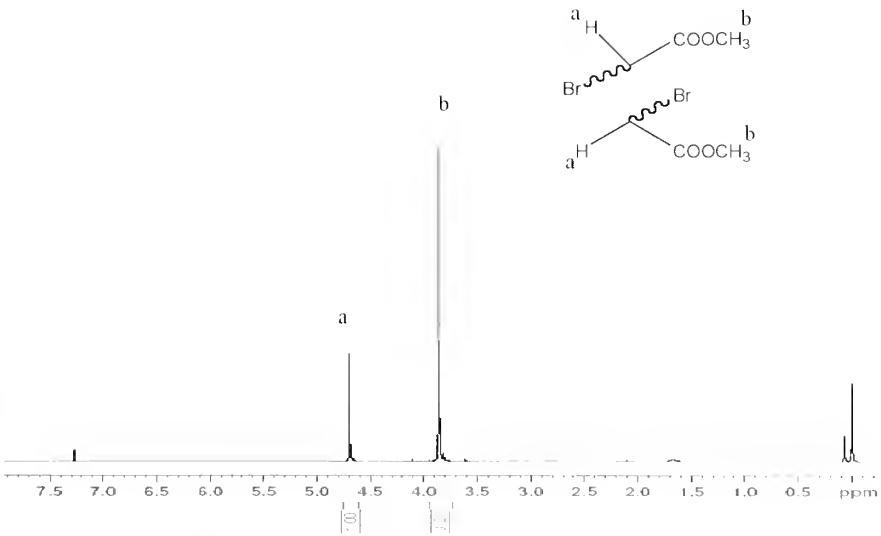


Fig 1. ^{1}H Nuclear Magnetic Resonance Spectrum of (±)-Dimethyl 2,3-dibromosuccinate.

When the reaction was performed using higher concentration solution (0.5 M) for the reaction mixture, only the isomerized trans product was formed in trace amounts with no addition reaction taking place in all three methods. With lower concentration (0.05 M) solution, addition or isomerized product formed in moderate amounts. This is probably because at lower concentration, molecules move around more freely, leading to a greater number of collisions between the substrate and the catalyst or reagent.

When the reaction was performed using Br_2 as a catalyst without adding any bromoform, only isomerized product was formed with no addition reaction taking place in all three methods. When bromoform was added as a catalyst in the absence of bromine, no reaction occurred even after 4 hrs. This suggests that bromoform has no effect as a catalyst upon treatment with maleic acid ester under the specified conditions. Hence, it should not have much effect on similar processes in the biological system if it is released into the environment in small amounts.

All reactions conducted under sunlight occurred at a faster rate, reaching equilibrium within one hour as observed from the color change and gave poorer yields compared to the other two methods. This is probably because the amount of heat transferred from sunlight to the reaction mixture in a given amount of time will be different and possibly less than from UV light.

Reactions were repeated for longer time period (24 hrs) under UV and reflux conditions to investigate the effect upon prolonged reaction times and also with larger amounts of bromoform with or without bromine. Once again interesting results were observed. When

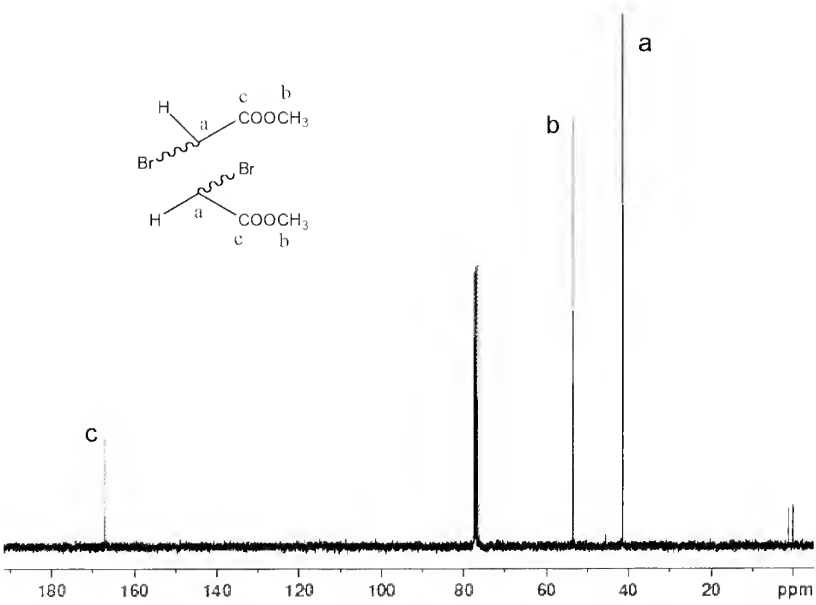
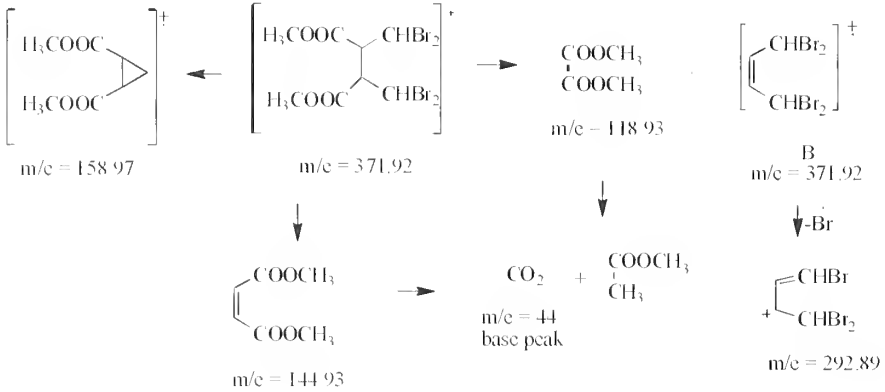


Fig2. ^{13}C Nuclear Magnetic Resonance Spectrum of (±)-Dimethyl 2,3-dibromosuccinate.

bromine was used as a reagent without any bromoform, only the anticipated addition product was obtained in maximum yield under both UV and reflux conditions. When only bromoform was used in equivalent amount as reagent without bromine, in CCl_4 solvent, after irradiation under UV for 24 hrs, white cubic crystals of the isomerized product (observed mp 95-99°C; lit. 102°C) were isolated in 11.2 % yield (^{1}H NMR, Fig 3) and no addition product formed.

The low yield indicated a slow reaction rate as anticipated. Prolonged reaction time did not improve the yield. The same reaction under reflux conditions resulted in no reaction. However, when the reaction was performed using DMME and bromoform in 1:3 ratio in CCl_4 solvent, without any added bromine, to our surprise, after 48 hrs of irradiation under UV, the reaction mixture changed from colorless to scarlet red indicating the possible release of bromine from bromoform via radical decomposition. Upon cooling, white crystals (mp 92-94°C) formed which was filtered and dried and characterized to be the trans-isomer, fumarate, from IR (a broad ester carbonyl band at 1700-1667 cm^{-1}) and NMR with only 85 % purity. The relatively low melting range compared to the reported value (lit. mp. 102°C), indicated the presence of other compounds as impurities. Further cooling of the mother liquor from the above filtration gave pale yellow crystals (mp 78-80°C) as second fraction in trace amounts. Both IR (C-H stretch at ~3000 cm^{-1}, broad ester carbonyl at ~1750 cm^{-1}, C-Br at ~522 cm^{-1}) and ^{1}H NMR (ppm: 3.75, m; 3.9, s; 6.7 s) spectral analyses indicate the possibility of the formation of compound A (Scheme 1) along with tetrabromoethane and other impurities as side products. Light-induced molecular rearrangement of bromoform to tetrabromoethane, bromine and other photochemical products and its role in ozone depletion has been reported earlier (Grecea *et al*, 2006). Photochemical conversion of halohydrins to ketones via oxidative decomposition and rearrangement has also been reported earlier (Piva, 1992). A mass spectrum of the crude product A (Fig 4a, 4b) showing the base peak at 44 m/e due to CO_2 fragmentation from the molecule and a peak at 371.92 m/e along with the M+2 (373.8), M+4 (375.9), M+6 (377.9) and M+8 (379.9) isotopic peaks corresponding to fragment B (Scheme 3) supports our proposed structure for A. Attempted recrystallization methods to isolate compound A in purer form were unsuccessful, and further characterization became impossible. However, when the reaction was repeated and continued for longer time (72-96 hrs), once again bromine release was observed after 48 hrs as light brown color, but the color disappeared upon continued irradiation. The solution color kept changing thereafter but with decreased intensity as the reaction continued. After 96 hrs of irradiation under UV, the reaction was stopped and excess solvent was evaporated under vacuum. Upon cooling, white crystals of the isomerized product, dimethyl fumarate, were obtained in better yield and purity. The product identity and purity were determined by melting point (102-3°C) and IR spectroscopy (Fig. 4a and 4b) using the commercial sample as control. The improved yield of the isomerized product could be due to the bromine released from bromoform, acting as a catalyst for further isomerization of the unreacted DMME and also from possible decomposition of compound A (Scheme 2). A mechanism for the formation of bromine and compound A and further isomerization is proposed and given in Scheme 2.



Scheme 3

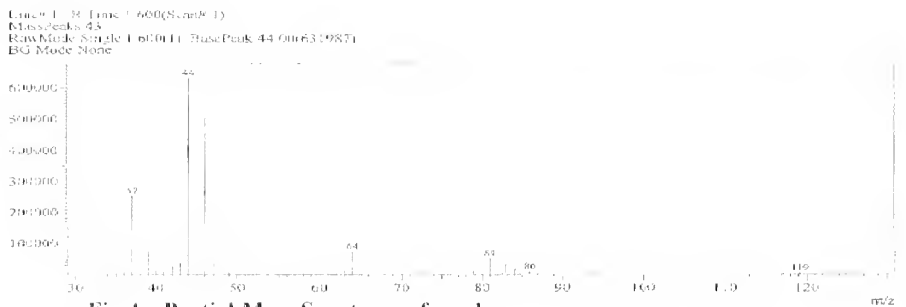


Fig 4a. Partial Mass Spectrum of crude A

Fig 4b. Partial Mass Spectrum of crude A

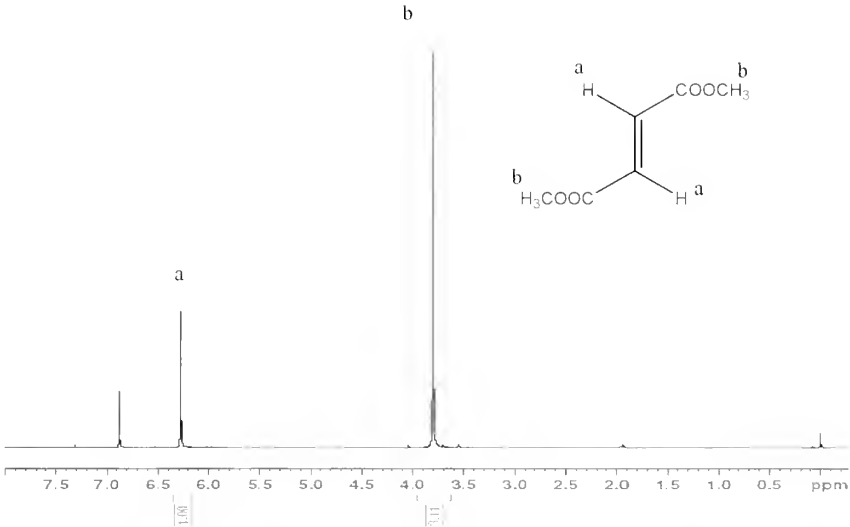


Fig 3. ^1H Nuclear Magnetic Resonance Spectrum of Dimethyl fumarate (DMFE)

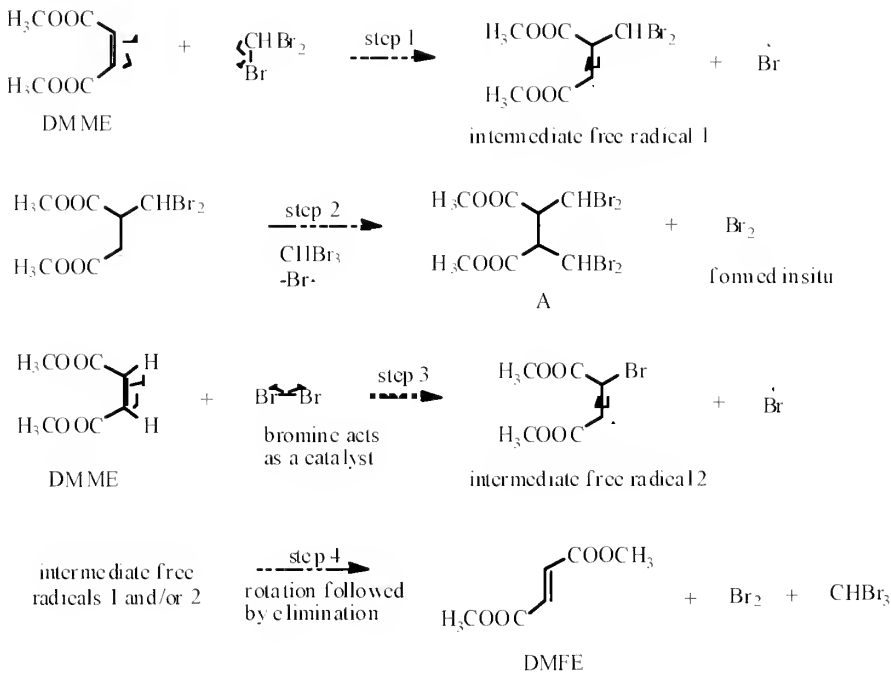
Fig 5a. FTIR spectrum of commercial dimethyl fumarate (DMFE).

Fig 5b. FTIR spectrum of dimethyl fumarate (DMFE) from the reaction of DMMF with $3CHBr_3$ isolated after irradiating under UV for 96 hrs.

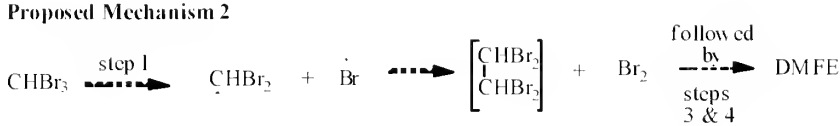
CONCLUSIONS

We successfully optimized the conditions for the synthesis of dimethyl 2,3-dibromosuccinate, the addition product, which was obtained selectively in 95 % yield and high purity by treating DMME with equivalent amount of bromine under reflux conditions. The formation of isomeric product also in high yield (95 %) but under prolonged

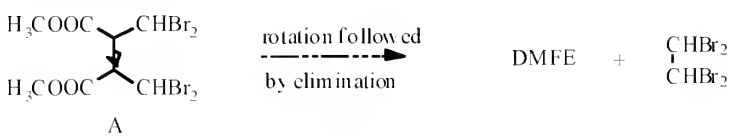
Proposed Mechanism 1



Proposed Mechanism 2



Proposed Mechanism 3



Scheme 2

UV irradiation in presence of excess bromoform infers that by optimizing the conditions, the selective synthesis of the meso-dibromo adduct can be achieved, which will be one of our future goals. Prolonged reaction time led to the formation of several intermediates including compound A, and a reaction mechanism has been proposed based on the data obtained. From these studies, we also conclude that trace amounts of bromoform released in the air from its commercial use and in research laboratories and industries should have minimal adverse effects on similar processes in biological systems. But, the release of large amounts may result in the isomerization process and release of bromine and other unwanted side products, which could have some adverse effects. The proposed mechanism (Scheme 2) for the formation of product A involving radical intermediates could also explain biotransformation of ingested halogenated compounds in organisms. Further exploration of addition versus isomerization reactions of DMME and DMFE under sunlight and UV light using other inorganic catalysts and enzyme catalysts on these reactions will be studied as part of our future research.

ACKNOWLEDGEMENTS

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AFTER THE DOUBLE HELIX. . . WHAT?

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ABSTRACT

When Watson and Crick puzzled out DNA's structure, everyone understood that science was poised to take a new direction. Watson and Crick knew that DNA controlled the creation of proteins, the building blocks of organisms, but did not understand how it worked. They and other researchers in the field were aware that RNA played a critical role, but, again, they did not understand the relation between DNA, RNA, and proteins. In many ways, the search for the answers to these questions is as fascinating and revealing as the search for the structure of DNA. They faced a problem that was completely new to science: How does one array of molecules somehow direct the assembly of a completely different set? They had to determine how to formulate the problem; only then would they be able to seek out the chemical processes that could provide the answer they needed. The search reveals a picture of talented, resourceful, and highly motivated people who struggled to formulate conceptions of the mechanism they sought, make decisions about which empirical data to seek, and try one idea after another until, with luck, hard work, and the passage of time, they puzzled out the answer.

INTRODUCTION

We are blind people, imagining what we don't see (Lightman, 2005, p. 64).

The story commonly ends when Francis Crick burst into the Eagle Pub and shouted, "We have found the secret of life!" (Hayes, 1998). The discovery of DNA's double helix structure is among the most critically important events in the history of science, and it forever changed the shape of biology. But the story of what happened after Watson and Crick's discovery is in many ways as compelling and revealing as their 1953 achievement. As Crick's triumphal announcement indicates, the small group of researchers in this area knew that DNA controlled the creation of proteins. At that time, some also suspected RNA played a role in this process. However, they understood neither how DNA and RNA were formed nor how they worked. So they began a frantic race to puzzle out the answers to these questions.

Their search helps illuminate one of the ways in which scientific knowledge progresses. Roald Hoffmann recently noted that the results published in journal articles give a distorted picture of the ways in which scientific advancement occurs. The narratives appearing in

professional journals commonly depict a series of steps that proceed in orderly fashion. The reality of scientific research is far different. Researchers drift into blind alleys, then feel their way back out. They are distracted by false theories or mistaken approaches. They try one thing after another, sometimes in desperation. In the end, and with luck, they may stumble onto the truth (Hoffmann, 2003). That complex and messy search is nicely illustrated by DNA's story.

THE ROLE OF METAPHOR

Part of the difficulty but also the allure of their quest is that the DNA researchers were grappling with an issue that was entirely new to science. There was no precedent for thinking about the way in which one array of inert molecules could somehow direct the creation of an entirely different array of equally inert molecules. Though this particular question is unprecedented, it is a compelling example of a broader problem that may be as old as science itself: How can we extend our thought to domains that have previously ranged beyond the scope of our understanding? At any given time, human beings have a set of concepts they use to fathom the world around them. But what happens when they strain to understand things that fall outside the reach of those ideas? How can we come to understand something we have never understood before and do not know how to imagine? Yet, the fundamental goal of all science is to push our understanding of the universe beyond its present boundaries. More often than not, this is simply a matter of dredging up new factual data. Scientists are keen to devise technologies that will allow them to see further or deeper in hopes that these technologies will reveal new factual data that will, in turn, deepen and extend our understanding. In typical cases, this new information will fit into the theories and ideas that we already possess.

From time to time, however, scientists confront an issue that cannot be grasped through traditional thinking. In such cases, they are compelled to stretch existing ideas to include material they cannot comprehend. As Alan Lightman recognizes, analogy is a primary means that scientists employ to expand their understanding to new domains (Lightman, 2005, pp. 49-64). This use of analogy likely reaches back to the ancient roots of science. Ernan McMullin has this to say:

> One [of the themes of ancient Greek thought] was that of *explaining*, of taking some feature of the world and making it more intelligible than it has been. This might be done by calling on simple analogies drawn from ordinary experience, the way in which eddies form in a river or mud settles in a lake, for example. *The less familiar would thus be explained by reference to something more familiar; what was less intelligible by invoking what was more intelligible. The function of explanation in these cases is to produce understanding.* . . (McMullin, 1989, p. 273) [emphasis added].

In this vein, I offer two simple ideas. First, scientists commonly seek to find a way to *imagine* new material before they can begin to understand it. Second, they must use analogy to do so.

So, it was perhaps inevitable that the post-double helix researchers would seek an

analogy that would allow them to imagine, if not (in the early stages of research) understand the way in which one set of inert molecules could direct the fabrication of an entirely different set of molecules. Watson and Crick were aware of several things that focused their thinking considerably. First, the helix itself, being double, suggests an obvious mechanism for replication: It can simply unzip. Second, they knew that nucleotides bonded to one another in specific fashion. Third, they were aware that the *sequence* of nucleotide bonds mattered a great deal. In addition, they understood that nucleotides were of four kinds (Watson and Crick, 1953). Briefly thereafter, they determined that bodily proteins were assembled from 20 amino acids (Crick, 1988, pp. 91-92). And, of course, they understood there had to be a connection of some sort between the four nucleotides and the 20 amino acid types.

In this particular case, however, the choice of analogy may have been nearly foreordained. Erwin Schrödinger's 1944 book *What is Life?* referred to the genome as a "code-script" (Schrödinger, 1944, p. 22). Of course, Schrödinger's work appeared amid the Second World War, a period of intense code making and code breaking. Codes were in the atmosphere, and many of the most talented scientists of the era worked with them. But, in addition, something equally epochal happened in the years following the war. In 1948 Claude Shannon's "A Mathematical Theory of Information" appeared in the *Bell System Technical Journal.* Shannon's theory cemented in place the ideas required to continue the development of computers as information storage devices. But, for our purposes, the crucial factor is that Shannon introduced the idea that nearly anything can be treated as information (Kay, 2000, pp. 128-135).

THE RNA TIE CLUB

As it happened, the deciding factor burst into the tight circle of DNA researchers in the guise of the ebullient, émigré Russian physicist, prankster, military consultant, and near alcoholic, George Gamow. Gamow had been heavily engaged with coding during the war and remained in regular contact with the military in the 1950's. When visiting a laboratory at Berkeley, he encountered Luis Alvarez, who excitedly showed him a copy of Watson and Crick's article. Gamow glanced through it and was fascinated (Kay, 2000, p. 131). He soon wrote to Watson and Crick, who dismissed him as a crank. But, Gamow was not easily dismissed, and his first letter contained the following thought: "If your [i.e., Watson and Crick's] view is correct each organism will be characterized by a long number written in quadrucal (?) system with figures 1,2,3,4 standing for different bases. . . This would open a very exciting possibility of theoretical research based on combinatorix [sic] and the theory of numbers!" (Kay, 2000, pp. 131-132). Gamow eventually met Crick in person and favorably impressed him with his intelligence and energy. But, as Crick noted, Gamow transformed their research. Crick puts the matter this way: "Gamow had made another contribution. We eventually realized that solving the code could be viewed as an abstract problem, divorced from the actual biochemical details. Perhaps by studying the restrictions on the amino acid sequences, as they became available, and by watching how

mutants affected a particular sequence, one could crack the code without having to know all the intervening biochemical steps. Such an approach seems natural to a physieist. . ." (Crick, 1988, p. 93). In this frame of mind, Gamow soon devised a code, which eame to be called the Diamond Code. Largely innoeent of biology, Gamow presumed that the genetic code would reside in the physical configuration of the surfaee of the double helix. He believed its diamond-shaped voids could provide docking points for the molecules required to assemble amino acids. Francis Crick soon dismantled Gamow's idea, but the notion that the double helix should be viewed as a code—to be eracked by intrepid researchers—remained (Kay, 2000, pp. 136-139).

Soon thereafter, Gamow fell in with a group of researehers who convinced him that RNA played the role of transmitting genetic information from the double helix to an area outside the nucleus of the cell where amino acids are assembled into protein. With his usual exuberance and impish humor, Gamow then assembled a loose group of researchers into the RNA Tie Club. It was limited to 20 members, one for each amino acid, and 4 honorary members, one for each nucleotide. The members proceeded to exchange papers, ideas, and criticism with one another. The group assembled by Gamow consisted mostly of physical seientists, and they were enthralled by the prospect of creating codes (Kay, 2000, p. 113). So for the next several years, they devised a riot of codes, many ingenious, some quite beautiful, and all of them wrong.

THE CODE

In the end, the code was cracked in the government-issue National Institutes of Health laboratory of Marshall Nirenberg and Heinrich Matthaei in 1961. They were not members of the tightly-knit group of RNA Tie Club enthusiasts, and they worked largely out of the publie eye. They employed a eell-free medium, which is basically a soup of the innards of *E. coli* cells whieh have been shorn of their cell walls. The DNA in the medium was inactivated so that it could produce no proteins. Next, they secured samples of RNA in various configurations and added them to this eell-free medium. After several false starts, they added a triplet of uracil (i.e., triple U or polyuridylic acid) to the cell-free medium. After a bit, they were elated to discover the amino acid phenylalanine. Ever rigorous, they took measures to insure that phenylalanine was produced in the cell-free medium only after polyuridylic acid was present. At that point, Nirenberg and Matthaei recognized that the triplet of uracil was the material that directed production of phenylalanine. They eoneluded, "The results indicate that polyuridylie acid contains the information for the synthesis of a protein having many of the characteristics of poly-L-phenylalaine" (Nirenberg and Matthaei, 1961, p. 1601). Their discovery turned the tide. By 1966, the complete RNA code for all 20 amino acids was in hand (Regis, 2007). What is more, to add insult to injury, "The code resembled none of the theoretical notions " (Hayes, 1998).

So, were the years of chasing after codes a waste, and the codes another scientifie blind alley? It is possible that Francis Crick believes so, for in his memoir *What Mad Pursuit*, he bemoans the exuberant theorizing of the RNA Tie Club era. In fact, there is

some reason to believe that the matter continued to weigh on him, for he offers a number of perceptive reflections on the differences between biology and physics (Crick, 1988, pp. 137-140). Others, even skeptical observers of the RNA Tie Club era, are not so sure. One recent commentator notes that the antic coding of the 1950's provided Marshall Nirenberg with the conceptual apparatus necessary to grasp the problem he was laboring to solve. Nirenberg himself referred to the genome as a code (Kay, quoting Carl Woese, 2000, pp. 123-124). And of course, the language of codes remains firmly attached to discussion of the genome.

The enthusiastic but futile theorizing of Crick and his associates aside, was the code metaphor useful for understanding DNA's operation? According to Alan Lightman, the lure of metaphors is nearly irresistible, even in the most abstruse physical investigations of phenomena which can never be experienced by human senses. As he puts it, "In doing science, . . . it is almost impossible not to reason by physical analogy, not to form mental pictures. . ." (Lightman, 2005, p. 50). Metaphors beguile, according to Lightman, because scientists are human beings who are driven to imagine the objects of their research even if they can never experience them directly. And if they are to imagine them, they must do so by employing analogies with things they can experience directly. Hence, they resort to metaphor. Nonetheless, it is certainly possible for metaphors to be ill-chosen, either because they give a wildly mistaken image of the research topic or because they are not fruitful, in the sense that they do not suggest avenues for further investigation. Nonetheless, the matter is complex. As Lightman notes, sometimes a metaphor can be highly fruitful even though wrong. James Clerk Maxwell, while working out his definitive theories of electricity, thought of its flow in strictly mechanical fashion. That mechanical picture is wrong, but, as Alan Lightman points out, "It led him to the correct equations" (Lightman, 2005, p. 55).

So, what makes a metaphor good? Steven Pinker, a distinguished researcher and widely-read author, enjoys uncommon facility in both science and language. He observes, "A good analogy helps you think: the more you ponder it, the better you understand the phenomenon" (Pinker, 2007). Another astute observer of science, Ernan McMullin, makes much the same point. He says, "A good metaphor allows the imagination to work, and guides it in certain directions. It is not a merely vague or indefinite object of thought" (McMullin, 1976, p. 427). If a metaphor is not fruitful in these ways or directs thinking away from relevant issues, it is bad. In both these ways, the code metaphor was highly fruitful. It indeed prompted researchers to look carefully at DNA, seek meaning in the sequences of its nucleotides, and try to grasp their connections with amino acids.

But likely the most successful metaphor is one that eventually destroys itself by ceasing to be a metaphor. And, as Hans-Jörg Rheinberger noted recently, that is precisely what happened with DNA. Rheinberger observed in 2000, "The laboratory reality of an organism now is no longer represented by, or embodied in, texts, but rather is an instantiation of a text" (Rheinberger, 2000, p. 128). Or, according to Lily Kay, "That these scriptural representations did not serve for Nirenberg as a rhetorical veneer but formed conceptual structures shaping the experimental practice is abundantly evident from the entries in

Nirenberg's work diaries in the fall of 1961" (Kay, 2000, p. 264). In other words, DNA has *become* a code, albeit a code which can be read. Moreover, it is a code able to reveal new meaning. For example, the great bulk of DNA does not code for amino acids. Previously, these non-coding sectors of the genome were termed "junk DNA," both because they could not be read and because it was believed they had no message for a would-be reader. Now, however, researchers are learning to read some portions of this "junk," and it is compelling reading. They have found that these sections indeed code—but they code for what is called micro-RNA. Micro-RNA does not direct the creation of amino acids. Rather, it plays an essential controlling role. It determines which genes are active and which are silenced, for example (Clancy, 2008). Hence, not merely can these sectors be read, but the information they contain is exceedingly important.

We can nonetheless ask whether metaphor, however useful, is absolutely essential for scientific advance. Werner Heisenberg apparently thought it was not. In fact, he believed it was counterproductive. He asserted, "Quantum mechanics has above all to free itself from those intuitive pictures" (Lightman, 2005, p. 60). On this matter, he clashed with Ernst Schrödinger, an enthusiastic partisan of metaphor. Schrödinger responded that he "felt repelled by the methods of transcendental algebra" (Lightman, 2005, p. 60). We note that a considerable portion of Einstein's genius lay in his eager exploitation of vivid metaphors. It is highly unlikely that he would have been able to make his discoveries without his inspired visions. On the other hand, Einstein's nemesis, quantum mechanics, mightily resists all attempts at imaginative grasp via metaphor. Had Bohr insisted on imaginative imagery to guide his thought, he would likely have failed (Lightman, 2005, p. 61).

So no clear picture emerges. In some cases, and in certain types of problems, metaphor is an essential tool for the researcher. In other cases and other problems, it appears to be irrelevant and perhaps a hindrance. But what is it that allows researchers to sometimes escape the lure of metaphor? The answer appears to be the "transcendental algebra" that offended Schrödinger. But mathematics, too, has a significant limitation. There are certainly cases where it is a necessary tool for scientific progress. Nonetheless, without metaphorical imagery, scientists gain no sense of understanding. Richard Feyman (a member of the RNA Tie Club, by the way, and amino acid GLY—Watson, 2003, p. 70) is well aware of this, but cheerfully acknowledges the lack. Regarding quantum electrodynamics, he confesses that, "You see my students don't understand it either. That is because *I* don't understand it. Nobody does" (Feyman, 1988, p. 9). His equations work, and that's enough for him (Feyman, 1988, pp. 9-10).

So mathematics is another way to stretch the grasp of science. But would this option have been of use to the DNA researchers? That is unlikely. As Crick appears to understand, the mistake the RNA Tie Club members made was to fail to understand the differences between biology and physics. The code analogy did not lead them astray. Instead, the difficulty is that their codes were too mathematical. A second aspect of their mistake was fixating on codes alone without exploiting the metaphor to inspire further thought and research. As it happened, Marshall Nirenberg and Heinrich Matthaei avoided abstract code construction, went to work in the laboratory, and, as we know, were successful while the others failed.

FINAL NOTES

This story has a couple of additional twists. As researchers eventually discovered, DNA is more complex, more subtle, and far less elegant than the codes concocted by the RNA Tie Club. Its code is also far more robust. The Tie Club codes are highly intolerant of error, while the DNA's code, inelegant with its repetitions and lack of precision, can accommodate a fair amount of transcription error. So organisms that rely on DNA to sustain existence are fortunate that it is coded as it is. One contemporary observer remarks that, "These observations suggest that I should be grateful my genes were not designed by George Gamow or Francis Crick. With Gamow's overlapping codes, any mutation could alter three adjacent amino acids at once, possibly disabling the protein. Comma-free codes are even more brittle in this respect, since a mutated codon is likely to become nonsense and terminate the translation" (Hayes, 1998). But in a final twist, researchers are presently investigating ways to employ DNA to both break and create codes (KentuckyFC, 2009). So the code metaphor has come nearly full circle.

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